



DRIED SPOT CARDS TO ANALYSE BIOLOGIC FLUIDS FOR DIAGNOSTIC INVESTIGATION OF PATIENTS

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DECLARATION

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Signature

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Date

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ABBREVIATIONS

BHT	- Butylated Hydroxytoluene
BSA	- Bovine Serum Albumin
CD(s)	- Conjugated diene (s)
CDC	- Centers for Disease Control and Prevention
cm	- Centimetres
CVD	- Cardiovascular disease
DAOS	- Dimethoxyaniline sodium salt
DBS	- Dried blood spot (s)
DBCS	- Dried Buffy Coat Spot (s)
DPS	- Dried plasma Spot (s)
DHC	- 7-dehydrocholesterol
dH ₂ O	- Deionized water
DNA	- Deoxyribonucleic acid
DUS	- Dried Urine Spot
EDTA	- Ethylenediaminetetraacetic acid
FC	- Folin Ciocalteu
FH	- Familial Hypercholesterolemia
GC-MS	- Gas chromatography-mass spectrometry
GK	- Glycerol kinase
GOD	- Glucose oxidase
GPO	- Glycerol-3-phosphate oxidase
HCN	- Hydrogen cyanide
IEM	- Inborn Error(s) of Metabolism
LPL	- Lipoprotein lipase
MS	- Mass spectrometry
NaClO	- Sodium hypochlorite
NCD	- Non-communicable disease(s)
NSQAP	- Newborn Screening Quality Assurance Program
PAGE	- Polyacrylamide Gel Electrophoresis
PCR	- Polymerase Chain Reaction
PKU	- Phenylketonuria
POD	- Peroxidase
PUFA	- Polyunsaturated fatty acid
TBARS	- Thiobarbituric Acid Reactive Substance (s)

TEMED	- Tetramethylethylenediamine
TC	- Total cholesterol
TG	- Triacylglycerol/ Triglyceride (s)
TLC	- Thin Layer Chromatography
TOOS	- N-ethyl-N-(2 hydroxy-3-sulphopropyl) -m-toluidine
T2DM	- Type 2 Diabetes Mellitus
SD	- Standard Deviation
SDS	- Sodium Dodecyl Sulphate
µg	- Microgram(s)
µg/µL	- Microgram per microliter

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ABSTRACT

Background: Collection of biologic fluid for laboratory analysis requires relatively large samples, often with additives, and transport in fragile tubes. The analytes or matrices may be unstable so testing needs to be carried out quickly. Collection of these biologic fluids and drying them on filter paper can lower the cost of transporting the sample to the laboratory, avoid instability of the matrix, and degradation of the analytes.

Aim: The aim of this project was to develop an inexpensive, convenient, comprehensive and reproducible patient sample collection system which ensures integrity and ease of transport of small-scale samples at room temperature, as well as ensuring convenient long-term storage for subsequent analysis.

Methods: Samples (blood, buffy coat, serum, plasma and urine) were collected into various tubes and spotted onto filter paper cards. Concentrations of total cholesterol, triglyceride, phospholipids, glucose, lactate, and protein were measured in the original sample and dried plasma spots (DPS) and the concentration of creatinine was measured in urine and dried urine spots (DUS). Determination of oxidation of lipids by measurement of conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) on dried serum spots (DSS) was carried out. Determination of salicylate on serum and dried serum spots and cyanide on whole blood and dried blood spots was carried out. Values obtained from original samples and dried spots were compared. In addition, DNA extracted from a dried buffy coat spot (DBCS) from a familial hypercholesterolemia patient was analysed after spotting.

Results: The total cholesterol, triglyceride, phospholipid, glucose, lactate and protein concentration values of 14 samples were compared in whole plasma and DPS stored at different temperatures. These were highly correlated after 1 week and 3 months of collection and storage. Plasma cholesterol, glucose and lactate concentration values for DPS as well as urinary creatinine for DUS at 1 week were not significantly different to that at both 3 and 7 months' analyses ($p > 0.05$). Plasma triglyceride and phospholipid concentrations were significantly different ($p < 0.0001$) between 1 week and 7 months. PCR analysis was successful for DNA extracted from DBCS stored at room temperature. CD and TBARS in DSS on the filter paper without BHT were significantly increased after 1 week of storage ($p < 0.05$), with significant reduction of TBARS after 3 months ($p < 0.05$). BHT prevented an increase in CD from baseline to 3 months. Mean extraction yields were 97% (5.236 ± 2.361 vs 5.425 ± 2.501 , whole serum vs DSS respectively) for salicylate and 92% (20.86 ± 7.576 vs 22.44 ± 7.3 , whole

blood vs DBS respectively) for cyanide. Salicylate in DSS and cyanide in DBS were not significantly different to the original samples (paired t-test, $p>0.05$).

Conclusion: Dried filter spots may be used to transport and store biologic fluid samples for analyses of a number of water-soluble and water-insoluble analytes. To protect lipids from being oxidised, the filter paper should be pre-treated with BHT.

1. LITERATURE REVIEW

1.1. Introduction

Biologic chemistry comprises numerous molecules that can be assayed, with chemical assays spanning analysis of liquids, tissues and gases. Commonest in practice are blood and urine but also stool, cerebrospinal fluid, and effusions of serosal spaces. Some deviations from the norm reflect changes affecting homeostasis adversely during clinical presentation, but others can predict clinical presentation later. While biochemistry reflects the metabolome, microbiome, xenobiome, and toxicome the genome is also of interest in its determination of metabolome and responses to other influences (Bloom N, 1989, Schwartzman R.A and Cidlowski J.A, 1993 and Cody R.S et al., 2005). Sample processing at the laboratory is typically divided into pre-analytical (receiving the sample and forwarding for analysis), analytical and post-analytical (interpreting and reporting) (Eising S et al., 2007 and Koster R.A et al., 2015). The clinician is typically involved at the pre-preanalytical stage and this is in relation to presentation of illness, epidemiology or research. While there may be a focus on a particular test, other tests may have to follow, especially in metabolic disease for which neonatal screening has become routine.

In this setting, sensitive tests are applied to small samples, with collection of urine from neonates mostly at home, onto cards to be sent to laboratories with high throughput, using TLC and GC-MS (Auray-Blais C et al., 2007).

1.2. Collection of blood samples

Currently, collection of samples for laboratory analysis of whole blood, plasma, serum and urine requires sampling in fragile tubes, usually with additives, with volumes ranging from 1-10 mL. Common additives include ethylenediaminetetraacetic acid (EDTA) or citrate (Tuck M.K et al., 2009). The analytes or the matrix may be unstable, so testing needs to be carried out relatively quickly. This requires prompt or special transport on ice by vehicle or courier services, in order to maintain sample integrity. Usually, in diagnostic laboratories, test tubes impose storage and time constraints which would be overcome by receiving samples on the proposed card system for this project for a variety of fluids in the appropriate matrix for analysis (Tuck M.K et al., 2009; Arzoumanian L, 2002; Magee L.S, 2005, Rai A.J et al., 2002 and Rai A.J et al., 2005). Gaseous and solid samples cannot be applied to the cards but liquids can. Although enzymes are no longer likely to be active in dried spots, oxygen and moisture might still affect the sample.

The samples derived from blood commonly used in most assays in clinical chemistry laboratories, are plasma or serum (Arzoumanian L, 2002). Plasma is obtained after centrifugation from blood collected in vacutainer tubes containing an anticoagulant (for example sodium or lithium heparin, EDTA, citrate, oxalate) while serum is obtained after coagulation and centrifugation without anticoagulant in the collection tube (Magee L.S, 2005, Rai A.J et al., 2002 and Rai A.J, 2005). Whole blood is rarely used in clinical laboratories for routine metabolic analysis (Sacks D.B et al., 2011).

The collection of biologic fluids onto filter paper is already in use (Lakshmy R et al., 2010 and Guthrie R and Susi A, 1963). It is important to distinguish between liquid specimens and dried biological specimens from filter paper card regardless of whether they are used in clinical or newborn screening assays. Analysing liquids is quite different from working with dried specimens, especially as the volume sampled needs to be taken into account, and quantitative approaches are needed to accurately determine the concentration of the metabolite or biomarker measured. A typical volume of blood collected by a phlebotomist ranges from 5 mL to 10 mL. Blood or plasma samples are sent either internally to a hospital-based laboratory or externally to a commercial specialty/reference laboratory. A typical volume of plasma undergoing analysis is mostly between 100 μ L to 500 μ L (Baker R.B et al., 2013). Blood from patients other than newborn babies is taken through venipuncture into the tube with or without additive depending on which samples need to be spotted on the filter paper card. In order to spot serum or plasma, blood is centrifuged and then spotted onto the filter paper with a pipette. For newborn screening, blood applied to a DBS card comes from a free flowing droplet that forms on the heel of an infant after lancing. The paper is applied to the outer surface of the drop that formed on the heel, which leads to the absorption of blood onto the paper. For convenience, the filter paper is marked with dashed circles to provide a target and an approximate volume of application.

Scope of the dried spot investigations

- Chemistry -
 - Inorganic (calcium, sodium, potassium and phosphate)
 - Organic (small molecules amino acids, lipids and carbohydrates)
 - Organic (proteins)
 - Organic (enzymes)
 - Toxicology (salicylate and cyanide)
 - Pharmacology
- Genetic

- Infection
 - Serology
 - Microbial DNA

1.3. Dried blood spots

1.3.1. Definition

Dried blood spot biosampling is the spotting of blood samples onto filter paper, followed by drying of the sample (as shown in Figure 2). Such cards are light and can reach the laboratory through ordinary mail. Samples can easily be transported in an envelope to an analytical laboratory where analyses can be undertaken (Edelbroek P et al., 2009, Liu G et al., 2010 and Guthrie R and Susi A, 1963). Collection of biologic fluids on filter paper has gained momentum as an alternative way of transporting samples to laboratories for analysis of analytes of interest (Koal T et al., 2005, Hoogtanders K et al., 2007). The type of filter paper plays a major role in analyte adsorption and extraction (Chapman O.D, 1924). The filter paper should not contribute chemicals nor contain reactive moieties that can modify analytes. While some interaction may occur between analytes and the filter paper (Figure 1) during drying, the analyte should be completely accessible at extraction and/or during chemical reaction.

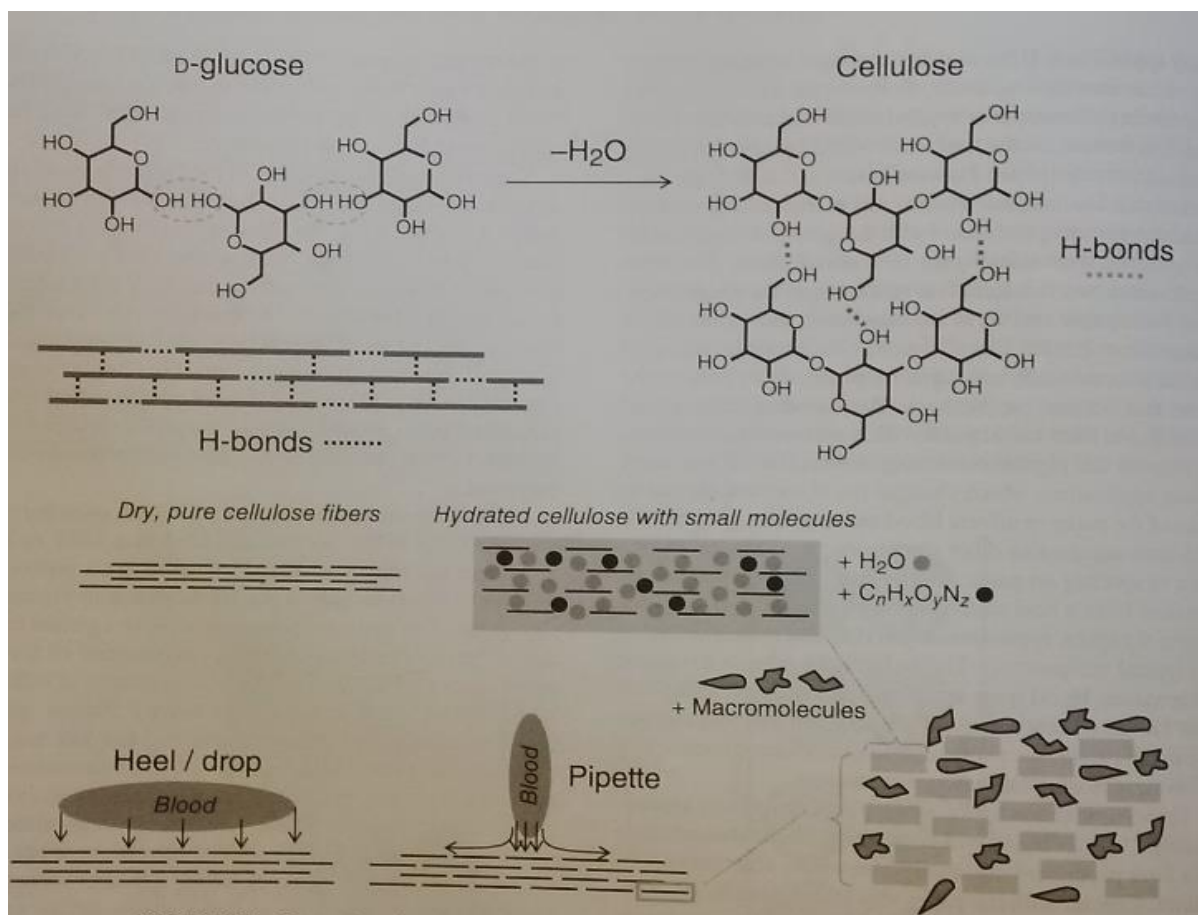


FIGURE 1: The use of filter paper showing the interaction of cellulose with the analytes in biologic fluids (copied from: Chance H.D et al., 2014)

1.3.2. History of dried spots

The concept of spotting whole blood onto filter paper was introduced more than a century ago by Ivar Bang when he used it to determine glucose concentration (Bang I.C, 1913). Since then, filter paper has been used more often as a scientific tool. Heatly in the 1940s described the use of filter paper for antimicrobial solutions, giving rise to the antibiotic susceptibility disc test (Heatly N.G, 1944). To overcome the difficulties in collecting blood for standard diagnostic tests under field conditions in Cuba, Chediak developed a method of identifying syphilis from dried blood on a glass slide (Chediak A, 1932). Zimmermann at the start of world War II in Germany adapted the method by drying finger or ear prick blood on strips of filter paper to diagnose syphilis using a microscopic agglutination test (Hannon W.H. and Therrell B.L., 2014). In 1950, Joe from Leiden (Netherlands) received faeces dried onto filter paper by post

from Indonesia and was able to detect shigella (Hannon W.H. and Therrell B.L., 2014). In 1961 Anderson published methods for detecting schistosoma antibodies in dried blood spots sent from endemic areas up to 3 months after collection (Anderson R.I. et al., 1961, Hannon W.H. and Therrell B.L., 2014).

Although dried biological fluids spots are relatively new to community research, their application has been available since the 1960s when Drs. Guthrie and Susi collected blood on filter paper for analysis of phenylalanine for diagnosis of phenylketonuria (PKU) (Guthrie and Susi, 1963). But it was Dr. Robert Guthrie who received the Nobel prize for introducing the use of filter paper for screening PKU in infants in Scotland (Guthrie R and Susi A, 1963). Since then filter paper has become a commonly used method for collection, storing and transporting diverse specimen types from humans, animals, and plants. Almost all types of human fluids have been spotted onto filter paper for a diverse range of biochemical tests (Lakshmy R and Gupta R 2009, Lakshmy R et al., 2010), for screening for genetic disorders, determination of metabolites by mass spectrometry (Tuchman M and McCann M.T, 1999), therapeutic drug monitoring and for detection of nucleic acids, and serological markers for infectious disease diagnosis.

1.3.3. Methodology of dried spots

For quantitative reasons, the volume of fluid used for spotting needs to be shown to be reproducible before and after drying following application. The area on which the liquid sample is dried should be accurately related to the volume of sample applied. The most challenging concept regarding DBS is the issue of volume of a liquid sample to dried spot. A punched disc from a dried blood specimen has a specific volume. Although it appears flat, a DBS is actually a disk defined by an circular area (πr^2) and a height (thickness) (h) with a volume. Given that the paper is thin and of even thickness, the volume of the original sample should yield a reproducible surface area. What is challenging is the association between this dried disk volume and a liquid volume counterpart. If the absorptivity of the paper is controlled, then volume equivalence between an actual liquid and dried specimen can be made. As a result, the volume of sample present in a punched disk will have a direct relationship to the surface area (mm^2). The Clinical and Laboratory Standards Institute (CLSI) has developed an approved standard (Hannon W.H et al., 2007) that describes the analytical process needed to determine how much serum is present in DBS punches prepared from whole blood at a normal haematocrit value.

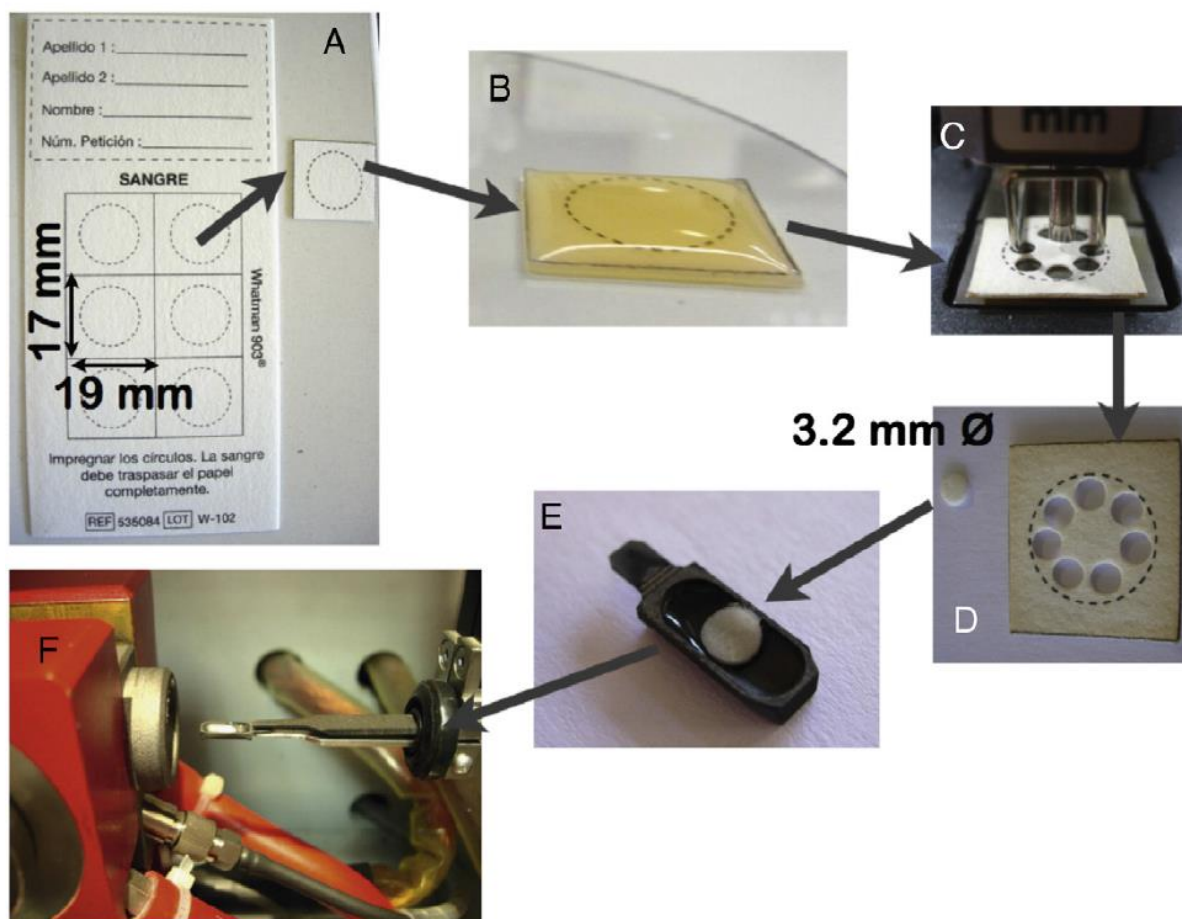


Figure 2: Illustration of punching the spot on filter paper with dried biologic fluids for analysis prior to elution of analytes (Rello L et al. 2013). A= circle marked where the sample will be spotted, B= spotted filter paper, C= punched filter paper spot for equivalent whole sample, D= filter paper card after punching out, E= punched blank spot and F= analysing sample after analyte(s) elution

1.3.4. Assays on dried blood spots

The first analytical assay for DBS was developed by Guthrie for detection of PKU using the bacteria which grow in the presence of phenylalanine, the marker for PKU. The bacterial inhibition assay (BIA) used bacteria that required phenylalanine for growth. The punched circle from the DBS was placed on agar containing specific bacteria. The presence of phenylalanine enabled the bacteria to grow, until it was consumed and the gel was stained to check the diameter of bacterial growth, which was equivalent to the level of phenylalanine in the blood (Guthrie R and Susi A, 1963). The method was simple and easily adapted by public health laboratories that had expertise in this type of assay. The BIA method was then adapted for

analysis of metabolites using the specific bacteria to determine metabolite of interest, such as leucine in detection of maple syrup urine disease (MSUD) (Naylor E.W and Guthrie R, 1978).

The filter paper sampling greatly simplified blood sample collection, handling, and storage over other methods in use at the time (Liu G et al., 2011, Liu G et al., 2010). Availability of analytically acceptable DBS has significantly impacted on a variety of fields for screening for various diseases (Kapur S et al., 2008). The use of DBS has increased over years due to ease of collection, and the technology for small volumes. As previously mentioned the first credit for using filter paper for whole blood collection is attributed to Bang after he introduced a method for spotting blood onto filter paper, drying, and extracting to determine glucose concentrations, although it was not published until 1913 (Bang I.C, 1913). The method of the spotting of blood for determination of glucose by Bang was practical and reliable. He also performed Kjeldahl nitrogen/ protein determinations with filter paper and was declared the founder of modern clinical chemistry by Schmidt (Schmidt R, 1986).

1.3.5. Chemical assays on dried plasma spots

Apart from screening for inborn errors of metabolism (IEM), mass screening of other analytes may be required. Some metabolic risk factors have gained attention as predictors of cardiovascular disease, type 2 diabetes mellitus, and other non-communicable disease (NCD) of morbidity and premature deaths (Buitrago-Lopez A et al. 2011, Castro J.P et al., 2003). The laboratory measurement of risk factors such as total cholesterol, triglycerides and glucose, for NCD helps prevention, monitoring and treatment of disease. The WHO recommends a stepwise approach for measuring markers of risk factors (WHO STEPwise).

1.3.6. Chemical assays on dried urine spots

Many small molecules are excreted in urine, some directly and some after conjugation. A study by Berry HK (1959) used filter paper to test phenylpyruvic acid, sugars, protein, and chondroitin sulphuric acid in urine dried on a filter paper spot. The use of dried urine spots for analysis of drugs and their metabolites has been shown to correlate with the original urine with lower coefficients of variation in DUS (Yamamoto AY et al., 2013). Other studies on biomonitoring have shown the advantages of using dried urine spots on filter paper compared to fresh urine samples. The study of Antunes et al has shown that analytes in urine are stable upon drying and the method saves a lot of money on the transportation (Antunes et al., 2013). Urine, compared to the other biologic fluids, is preferably used to confirm the use of illicit

substances (Rello L et al., 2013). This is mainly due to ease of collection and high levels of drugs and metabolites in comparison to the values found in serum.

1.3.7. Toxicology on dried spots

Forensic analysis has become one of the most fast-growing areas of bioanalytical chemistry in recent years (Brown S.D et al., 2007). The identification of analytes in various types of body fluids discovered at a crime scene is a major part of forensic investigation today. The increasing popularity of DNA analysis leaves investigators with a heavy burden to correctly identify an unknown material such as a specific body fluid without destroying the entire available sample. Fluids such as blood, semen, saliva, and vaginal secretion can be very useful in identifying a victim or suspect, and they can also help answer questions regarding the events of a crime. The ability to identify a particular body fluid quickly, easily, and non-destructively, as on dried filter paper from the scene of the crime would be a valuable tool for forensic investigators.

1.3.8. Genetic assays on dried buffy coat spots

Genetic disorders affecting metabolism of amino acids, lipids, carbohydrates, nucleic acids and proteins are individually rare but taken together are not rare (Wild et al., 2004). Except for patients presenting with acute and life-threatening developments that need immediate diagnostic investigation, it would be ideal for investigations to proceed from primary health care by an inexpensive mode that could provide for a range of biochemical and genetic tests. A wide range of different genetic disorders is currently known. Most of these disorders are diagnosed at birth or during early childhood, but some may present in adulthood. Early diagnosis and treatment may ameliorate the natural history (Antonarakis S.E, 1989). Once a biochemical alteration is detected, its genetic cause can be looked for. Some disorders may not show obvious biochemical alterations and could be detected by a genetic test.

2. PURPOSE OF THE STUDY:

2.1. AIM:

The aim of this study is to develop an inexpensive, convenient, comprehensive and reproducible patient sample collection system which ensures sample integrity and ease of transport of small scale samples at room temperature as well as ensuring convenient long-term storage for subsequent analysis.

2.2. OBJECTIVES:

- To establish the conditions for spotting a variety of biologic samples onto the card (whole blood, plasma, serum, buffy coat and urine) including assessing the properties of the filter paper for surface area to volume ratio
- To determine optimal drying times and conditions of samples on the card
- To check the efficacy of vacuum-sealing the card by comparing vacuum-sealed results with unsealed card results
- To check the stability of the analytes and reproducibility of the extracts from the card for the selected assays over a time period, both at ambient, fridge & freezer temperature
- To verify the suitability of the card extracts for conventional testing by comparing results with tests carried out on fresh samples and stored original samples
- To measure analytes by special techniques

3. MATERIALS AND METHODS

3.1. Ethical consideration

The study was approved by the UCT Research Ethics Committee (HREC REF: 673/2015). Pooled anonymous samples were used for the initial development of methods. Therefore studies were carried out on individual samples for each of the methods used. A consent form was signed by all subjects whose samples were used in this project.

3.2. Consent

Consent for obtaining samples for development, diagnosis and research was taken on an approved consent form (Appendix I).

3.3. Specimen collection

Blood samples were collected by conventional venepuncture into vacutainer tubes: ethylenediaminetetraacetic acid (EDTA) tubes for whole blood, plasma and buffy coat; and plain tubes without additive for serum. Biological samples, as mentioned in Objectives (Section 2.2.), were spotted on the filter paper card and dried in air at ambient temperature to minimise thermal damage and to mimic conditions that would pertain to most collection sites. Predetermined optimal volumes were used with appropriate drying times. To protect lipids from oxidation the filter paper was pre-soaked in 4 mmol/L butylated hydroxytoluene (BHT) (a known and well used antioxidant) in ethanol and dried prior to spotting the samples.

3.4. Filter paper

Sample Carrier Paper, Grade H182 182 g/m² (Dim.: 580 mm x 580 mm, Sartorius stedim biotechGmbH, Germany) was used in this study. This filter paper showed good consistency between the sample volume and surface area when spotted (as shown in Figure 16) and is cheap compared to Whatman 903.

3.5. Biological samples

Random biological samples from normal participants were used in this study, with the exception of the blood for DNA extraction, which included a sample from a consenting patient with familial hypercholesterolaemia (FH). To pilot the conditions for spotting onto cards, samples from 14 individuals were used. The original samples (including whole blood, plasma, serum, buffy coat and urine) were aliquoted into 1.5 mL microcentrifuge tube and stored at -20 °C.

3.6. Spotting of the samples on filter paper

3.6.1. Property of the paper

In this study, as mentioned in section 3.4. filter paper (Sample Carrier Paper, Grade H182) was used, which weighed 4.0 ± 0.7 mg for every 19.6 mm^2 area and this area absorbed $12.04 \pm 0.66 \mu\text{L}$ of dH_2O . The CV of the dried filter paper mass was 1.7 % and the CV for wet filter paper was 5.48 %.

3.6.2. Spreading of biologic samples

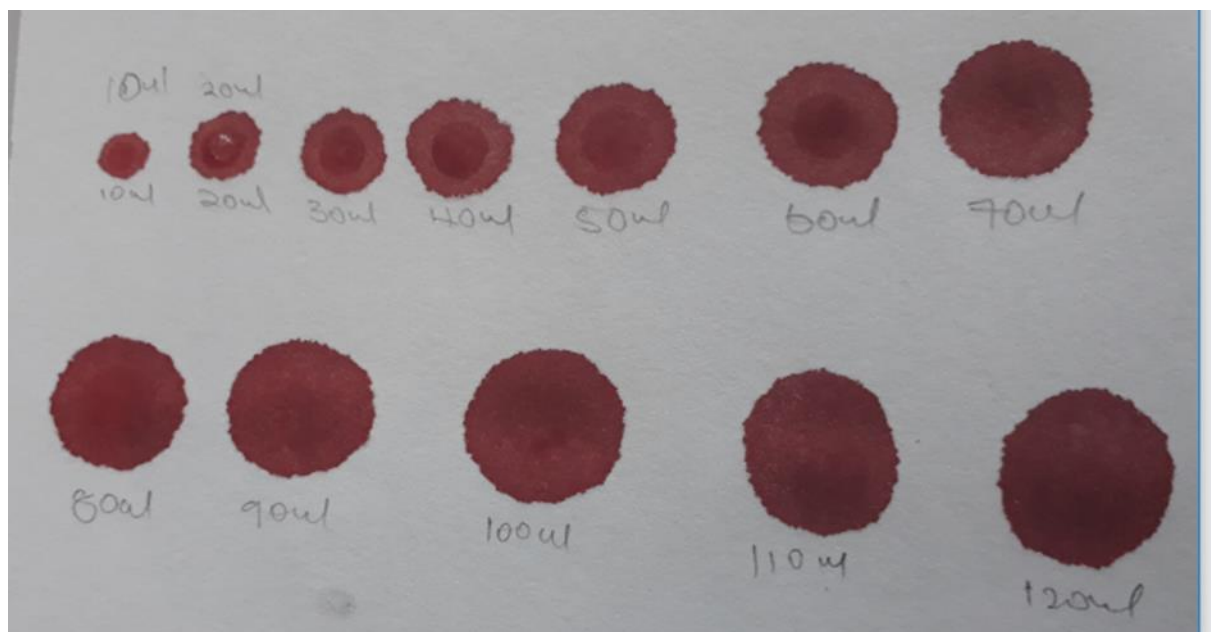


Figure 3: Relationship of spread of blood spotted on the filter paper to the volume of blood

The blood samples were taken by venesection into EDTA tubes, and mixed by inverting the tube gently. Tubes were centrifuged at 1000 x g for 15 minutes at room temperature to prepare serum and plasma. Serum was obtained from blood by using tubes without anticoagulants or additives and centrifuging at 1000 x g for 15 minutes. Whole blood was spotted onto filter paper to determine the surface areas of volumes ranging from 10 μ L to 120 μ L (Figure 3.). The same was done for serum, plasma and buffy coat. Thereafter a surface area to volume curve was drawn. Urine was collected into 50 mL tubes and spotted onto filter paper in volumes ranging from 5 μ L to 130 μ L and again a surface area to volume curve was drawn.

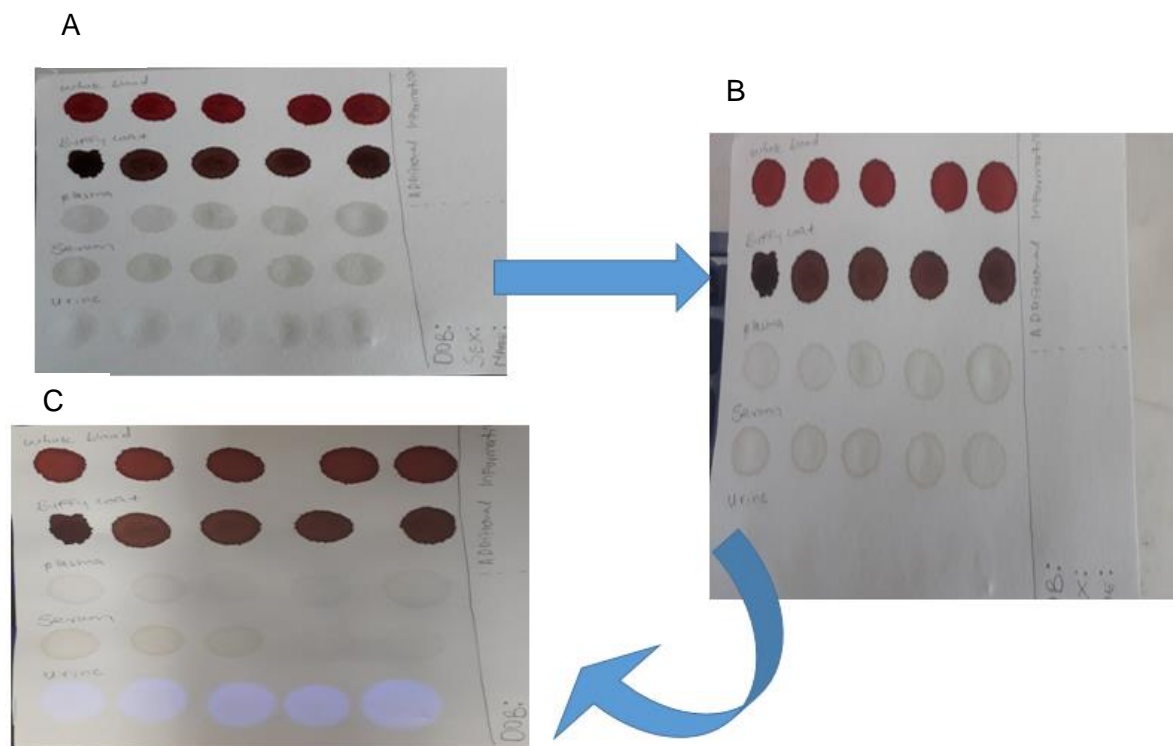


Figure 4: Spotting of the biologic fluids on the filter paper followed by UV illumination to demonstrate the urine spots. A= filter paper after spotting, with last row of urine still visible before drying, B= after the sample dried on the filter paper, the urine spot is poorly visible and C= visualising the dried urine spot under UV light

To determine the volume of serum and plasma to be used on the filter paper, 10 μ L to 150 μ L of each was spotted onto the filter paper. These resulted in irregular-shaped circles, and so in order to get an accurate surface area, transparent paper with regular squares was used to count the squares over the spots and then calculate the surface area of each spot. These surface areas were plotted against the spotted volumes for each biologic fluid.

It was checked which volume from these above-mentioned biologic fluids would create 15 mm diameter filter paper circles. For serum and plasma, it was 40 μ L, whole blood and buffy coat 60 μ L and urine 20 μ L (as shown in figure 4).

3.7. Drying times of samples on filter paper

The dry filter paper spot (15 mm diameter, Section 3.6.2. above) was weighed before biologic fluids (of each sample at specific volumes to fit into the 15 mm diameter circle) were spotted. After spotting, each paper spot was placed either onto a non-absorbent surface to dry, or was kept at 37 °C or 4 °C (to validate the drying time at those temperatures). To check for complete dryness, the spots were weighed at 30 minutes intervals over 3 hours. It was noted that the mass of the samples decreased until 3 hours, by which time the masses became unchanged (they were dry). This occurred at room temperature and 4 °C but at 37 °C after 2 hours the sample mass became constant.

3.8. Sealing of the filter paper

Dried filter paper spots with biologic fluids were sealed with a plastic seal which protected the samples from environmental influences such as moisture. A convenient commercially available device for home use was chosen as this might be affordable in developing countries such as South Africa. To determine the permeability of the plastic seal, cobalt chloride-impregnated silica crystals (cobalt chloride is purple without water but when hydrated is pink) and 6 rectangles of filter paper were heated at 90 °C for 4 hours to remove water, and then sealed within plastic pouches using a vacuum sealer (Genesis, Revolutionary Homeware). Duplicate sealed filter paper rectangles were immersed in a beaker filled with water, another 2 sealed filter papers with cobalt chloride were left at ambient temperature and another 2 sealed filter papers were placed in the refrigerator at 4 °C.

In addition, to determine the rate at which water leaves or enters the plastic seal, 2 ice cubes (and not water, for ease in sealing) were placed in duplicate plastic pouches and sealed. One sealed pouch was kept at ambient temperature and the other at 37 °C, both for a month. The mass of the pouches was measured daily to determine the change in mass of the water.

3.9. Laboratory tests

The order of analytes described below for this study follows the interest in Chemical Pathology: lipidology, inborn errors of metabolism (IEM), therapeutic monitoring, toxicology and genetic disorders

- I. Lipid analyses: total cholesterol (TC), triglyceride (TG) and phospholipids (PL) in plasma
- II. Routine analyte analyses: glucose, lactate, total protein and electrophoresis of proteins in plasma and creatinine in urine
- III. Therapeutic analysis: salicylate in serum
- IV. Toxicology analysis: cyanide in whole blood
- V. Research aspects of lipids: thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) in serum
- VI. Genetic analysis: polymerase chain reaction (PCR) in buffy coat

3.9.1. Determination of cholesterol concentration in plasma and dried plasma spots

Cholesterol concentration in plasma is determined by production, absorption and clearance rates of lipids in the liver and intestinal tract, and other organs.

The choline oxidase DAOS method spectrophotometric kit (LabAssay™cholesterol (Cholesterol Oxidase DAOS) (Wako Pure Chemical Industries, Ltd., Germany)) was used to determine the concentration of cholesterol in plasma and dried plasma spots. Cholesterol esters in the sample are decomposed into free cholesterol and fatty acids by cholesterol esterase. The cholesterol is oxidised by cholesterol oxidase, and simultaneously hydrogen peroxide is produced. The produced hydrogen peroxide reacts with DAOS, 4-Aminoantipyrin and a peroxidase (HRP), to produce a blue pigment. Quantitation of total cholesterol in the sample can be made by measurement of the absorbance at 600 nm wavelength.

3.9.1.1. Method for cholesterol concentration determination

Cholesterol is minimally soluble in water, but does dissolve slightly more in alcohol. Plasma cholesterol was measured by punching out two filter paper spots containing dried plasma equivalent to 7.4 μL of original sample. They were placed in a microcentrifuge tube and 100 μL of methanol was added. The sealed tube was heated for 10 minutes at 60 $^{\circ}\text{C}$. 25 μL of the liquid was pipetted into a well of a 96-well microtiter plate and 25 μL of dH_2O was added. 200 μL of Cholesterol Reagent was then added. The mixture was incubated for 20 minutes at 37 $^{\circ}\text{C}$ and the absorbance was read at 600 nm by spectrophotometry (Spectramax Plus 384, molecular devices, California, USA) using Softmax Pro (version 4.4) software. 7.4 μL of whole plasma was added to a separate Microcentrifuge tube and treated the same as above, with equal amounts of methanol in both reactions. The cholesterol concentration was derived from the linear regression of a cholesterol standard curve detailed in Appendix II (1) to compare the values of the direct (original samples) and indirect method (extracted from dried spot samples).

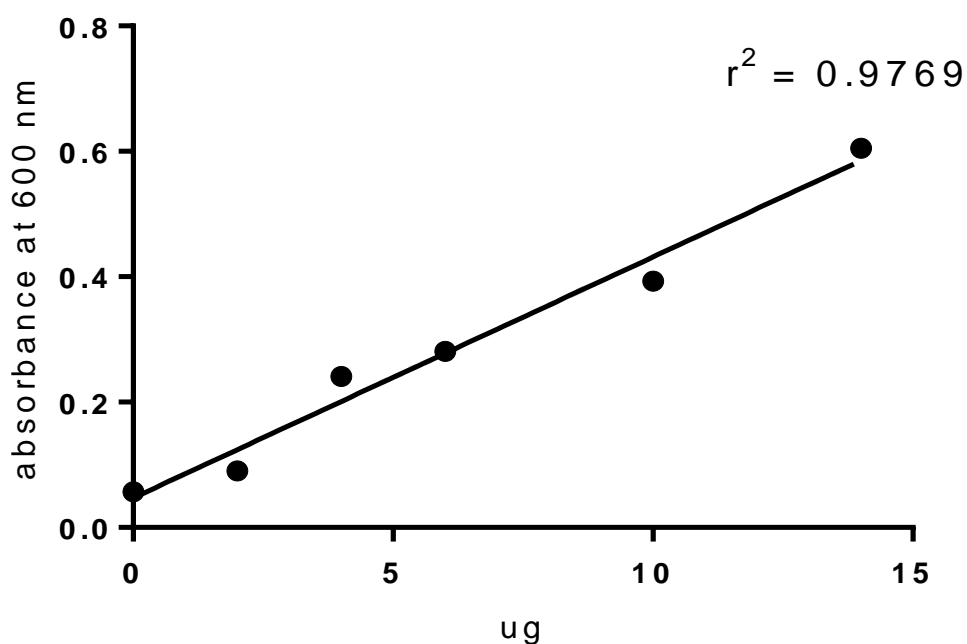


Figure 5: Standard calibration curve of cholesterol assay to determine the concentration of cholesterol in the dried plasma spots and whole plasma

3.9.2. Determination of triglyceride concentration in plasma and dried plasma spots

Triglycerides are insoluble in water but partially soluble in methanol. As major components of very low density lipoprotein and chylomicrons, triglycerides play an important role in metabolism as energy sources and transporters of dietary fat. The LabAssay kit for determining the concentration of triglycerides is based on enzymatic hydrolysis and using N-ethyl-N-(2-hydroxy-3-sulphopropyl)-3,5- dimethoxyaniline sodium salt to generate a blue colour, the absorbance of which is read spectrophotometrically at 600 nm.

The glycerol-3-phosphatase DAOS spectrophotometric kit (LabAssay™ Triglyceride (GPO DAOS method) (Wako Pure Chemical Industries, Ltd., Germany) was used to determine the concentration of triglycerides in original plasma and dried plasma spots. Triglycerides are hydrolysed to glycerol and free fatty acids in a reaction catalysed by lipoprotein lipase. Glycerol is converted to glycerol-3-phosphate which is oxidised by glycerol-3-phosphate oxidase in a reaction that produces hydrogen peroxide. The hydrogen peroxide causes DAOS and 4-aminoantipyrine to undergo quantitative oxidative condensation catalysed by peroxidase, producing a blue colour. The amount of triglyceride contained in the sample is determined by measuring the absorbance of the blue colour relative to the standard curve.

3.9.2.1. Method for triglyceride concentration determination

Two spots of filter paper containing dried plasma (equivalent to 7.4 µL of original plasma) were punched out and put inside an Microcentrifuge tube. 100 µL of methanol was added, and the tube was vortexed to mix the contents, and the sealed tube was incubated at 37 °C for 10 minutes. 25 µL was pipetted into a 96-well microtiter plate, 25 µL of saline and 200 µL of Triglyceride Reagent were added. The plate was incubated at 37 °C for 10 minutes and the absorbance was determined at 600 nm by spectrophotometry as described previously (Section 3.9.1.1.). 7.4 µL of whole plasma was added to a separate Microcentrifuge tube and treated the same as above, with equal amounts of methanol in both reactions. The triglyceride concentration was derived from the linear regression of a triglyceride standard curve with volumes detailed in Appendix II (2) to compare the values of the direct and indirect method.

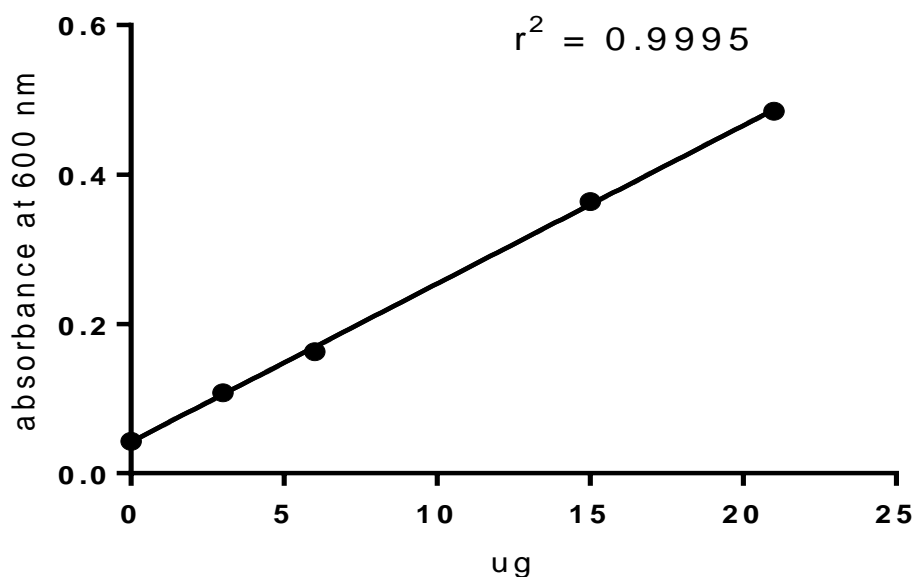


Figure 6: Standard calibration curve for triglyceride to determine the concentration of triglyceride in the dried plasma spots and whole plasma

3.9.3. Determination of phospholipid concentration in plasma and dried plasma spots

Phospholipids are a class of lipids, and a major component of all biological membranes, along with glycolipids and cholesterol. While phospholipids are not part of conventional plasma lipid investigation, they were investigated for complete understanding of lipid extraction.

The choline oxidase method spectrophotometric kit (LabAssay™ phospholipids Choline Oxidase DAOS kit (Wako Pure Chemical Industries, Ltd., Germany) was used to determine the concentration of phospholipids in plasma and dried plasma spots. Lecithin, sphingomyelin and lysolecithin in a sample are hydrolysed to choline in a reaction catalysed by phospholipase D. Choline is then oxidised by choline oxidase in a reaction that produces hydrogen peroxide. The hydrogen peroxide produced causes DAOS and 4-aminoantipyrine to undergo a quantitative oxidative condensation reaction catalysed by peroxidase (POD), producing a blue pigment. The concentration of phospholipid in the sample is determined by measuring the absorbance at 600 nm.

3.9.3.1. Method for phospholipid concentration determination

Two spots of filter paper containing dried plasma (equivalent to 7.4 μL of plasma) were punched out and put inside an Microcentrifuge tube. 100 μL of methanol was added, the tube was vortexed, and incubated at 37 $^{\circ}\text{C}$ for 10 minutes. 25 μL was pipetted into a 96-well microtiter plate, 25 μL of saline and 200 μL of Phospholipids Reagent were added. The plate was incubated at 37 $^{\circ}\text{C}$ for 10 minutes and the absorbance was determined at 600 nm by spectrophotometry as described previously (Section 3.9.1.1.). 7.4 μL of whole plasma were added to a separate Microcentrifuge tube and treated the same as above, with equal amounts of methanol in both reactions. The phospholipids concentration was derived from the linear regression of a phospholipids standard curve with volumes detailed in Appendix II (3).

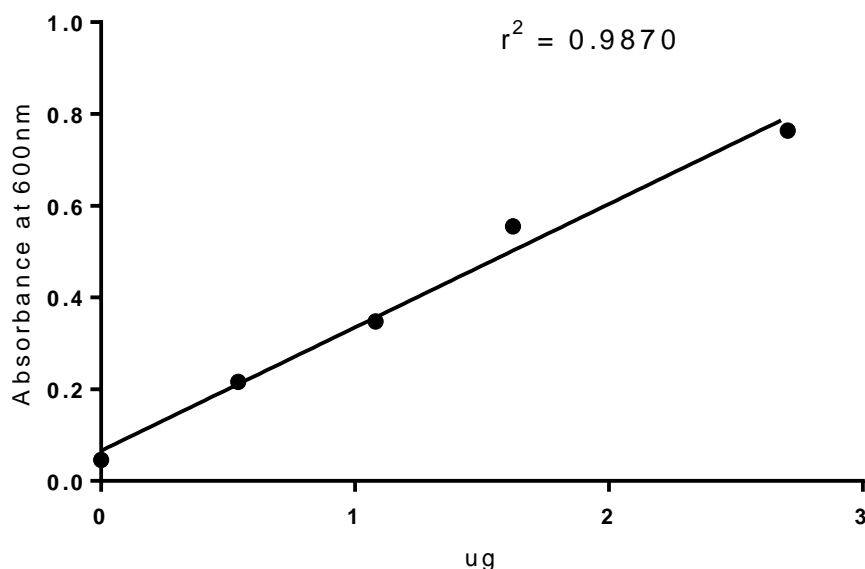


Figure 7: Standard calibration curve for phospholipid to determine the concentration of phospholipid in the dried plasma spots and whole plasma

3.9.4. Determination of glucose concentration in plasma and dried plasma spots

Glucose is a sugar molecule with the molecular formula $\text{C}_6\text{H}_{12}\text{O}_6$. The measurement of glucose in the blood is used to diagnose hyperglycaemia when the blood glucose rises above 5.6 mmol/L, and hypoglycaemia when blood glucose is below 2.9 mmol/L. The term diabetes

mellitus is used for severe hyperglycaemia, and reflects a metabolic state due to inability of the pancreas to secrete or produce insulin, or failure of insulin to bind its receptor or both (American Diabetes Association, 2004).

The mutarotase-GOD spectrophotometric kit (LabAssay™ Glucose Mutarotase-GOD method (Wako Pure Chemical Industries, Ltd., Germany) was used to determine the concentration of glucose in plasma and dried plasma spots. This assay involves the mixing of samples with the Chromogen Reagent supplied in the kit; the alpha-form of glucose in the sample is converted to the beta-form by mutarotase in the reagent. Beta-D-glucose then is oxidised by glucose oxidase to yield hydrogen peroxide. In the presence of peroxidase, the formed hydrogen peroxide yields a red colour by quantitative oxidation condensation with phenol and 4-aminoantipyrine. The glucose concentration is obtained by measuring the absorbance of the red colour and relating it to the standard curve.

3.9.4.1. Method for glucose concentration determination

Two spots of filter paper containing plasma (the equivalent of 7.4 µL plasma) were punched from the filter paper, placed into a 1.5 mL microcentrifuge tube and 100 µL of dH₂O was added. The tube was vortexed to mix the contents and incubated at 37 °C for 10 minutes. 20 µL was removed from the tube and added to a second 1.5 mL tube, to which 300 µL of Chromogen Reagent was added. A glucose set of standards from 0-10 µg was processed in the same way. The tubes were mixed gently and incubated at 37 °C for 5 minutes. 300 µL of the mixture was transferred to a 96-well microtiter plate, and the absorbance at 660 nm of the mixture was determined by spectrophotometry (Section 3.9.1.1.). 7.4 µL of whole plasma was added to a separate Microcentrifuge tube and treated the same as above. The glucose concentration was derived from the linear regression of a glucose standard curve with volumes detailed in Appendix II (4).

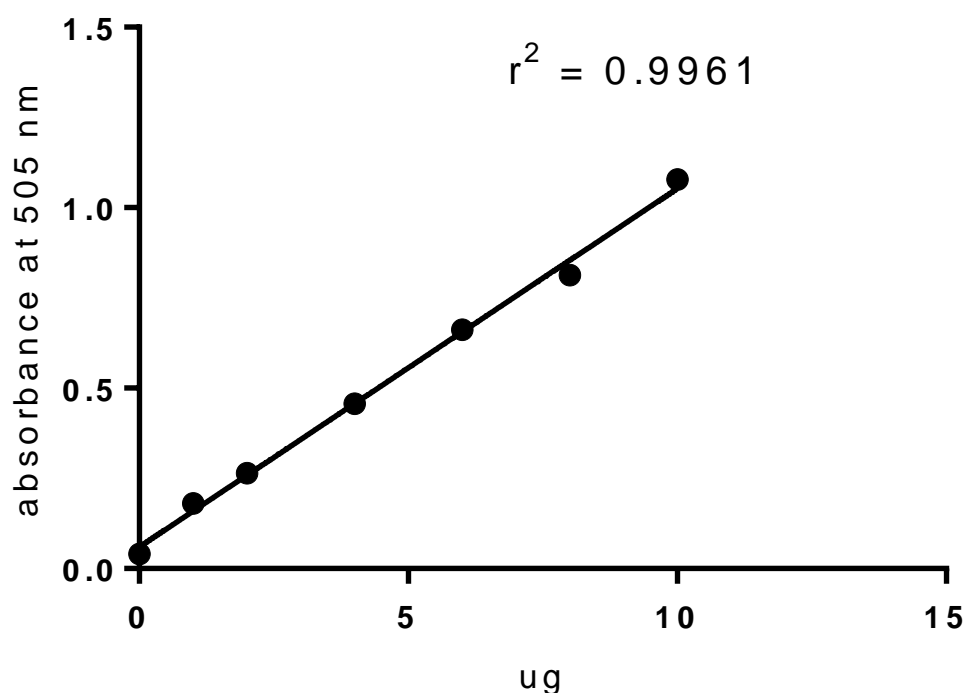


Figure 8: An example of a standard curve for glucose to determine the concentration of glucose in the dried plasma spots and whole plasma

3.9.5. Determination of lactate concentration in plasma and dried plasma spots

Aerobic glycolysis produces pyruvate which enters into TCA cycle but under anaerobic conditions or mitochondrial disease, pyruvate is converted to lactate. Lactate can revert to pyruvate or lactate can regenerate glucose in the liver (Cori cycle). Increased concentrations of lactate can be due to several causes ranging from poor perfusion to mitochondrial metabolic errors.

To determine the concentration of lactate in plasma, a commercialised enzymatic kit from RANDOX was used for quantitation of L-Lactate (Enzymatic determination of L-lactate manual (Randox Laboratories Limited, UK)). Lactate in the plasma is converted to pyruvate and hydrogen peroxide. The formed hydrogen peroxide combines with 4-aminoantipyrine and N-ethyl-N-(2 hydroxy-3-sulphopropyl)-m-toluidine (TOOS) by peroxidase to form a purple product. The purple product was measured by spectrophotometry at 550 nm.

3.9.5.1. Method for lactate concentration determination

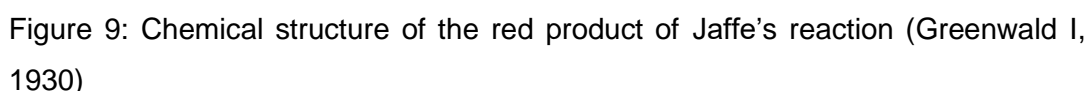
Two spots of filter paper containing dried plasma were punched out and placed in a 1.5 mL microcentrifuge tube. 100 µL of dH₂O was added, and the tube was vortexed thoroughly. 22.8 µL of the supernatant was pipetted into a 96-well microtiter plate and 200 µL of the Rib Reagent was added. The plate was incubated for 5 minutes at 37 °C and the absorbance was determined at 550 nm by spectrophotometry. 7.4 µL of whole plasma was added to a separate microcentrifuge tube and treated the same as above. The lactate concentration was calculated using the formula:

$$\text{L-lactate concentration} = (\text{A sample} / \text{A standard}) * 4.35\text{mmol/l}$$

(where A= absorbance at 550 nm)

3.9.6. Determination of creatinine concentration in urine and dried urine spots

Creatinine is a breakdown product of creatine phosphate in the muscle. It is mainly excreted passively by the kidneys, though a small amount is actively excreted. It is used to evaluate or monitor kidney function. The creatinine assay is based on an *in vitro* spectrophotometric kit (LabAssay™ Creatinine (Jaffe method) (Wako Pure Chemical Industries, Ltd., Germany)) for the quantitative determination of creatinine in urine. After adding a deproteinising reagent (to precipitate protein and lipids, see Figure 31) to the sample the supernatant is separated from a centrifuged to protein pellet. A classical spectrophotometric reaction used to determine the concentration of creatinine in urine is known as the Jaffe Reaction, and was first developed by Max Jaffe in 1886 (Jaffe M, 1886). Creatinine in the sample reacts with picric acid in an alkaline solution to produce an red colour.



Two spots of filter paper discs containing dried samples of urine (equivalent to 3.8 μL of urine) were punched and placed into a 1.5 mL microcentrifuge tube and 100 μL of dH_2O was added and vortexed to dissolve the analyte. The tube was incubated at 37 $^{\circ}\text{C}$ for 10 minutes. 20 μL of the mixture were transferred into a fresh microcentrifuge tube and 300 μL of Deproteinising Reagent were added, mixed well, stood at room temperature for 10 minutes and then centrifuged at 1000 $\times g$ for 10 minutes. 100 μL of the supernatant was transferred into a 96-well microtiter plate and 50 μL picric acid reagent, and 50 μL of 0.75 mol/L sodium hydroxide solution were added. This was kept at 25-30 $^{\circ}\text{C}$ for 20 minutes. The absorbance of the mixture was determined by spectrophotometry (as described previously in Section 3.9.1.1.) at 520 nm. 3.8 μL of whole urine were added to a separate microcentrifuge tube and treated the same as above. The creatinine concentration was derived from the linear regression of a creatinine standard curve with volumes detailed in Appendix II (5).

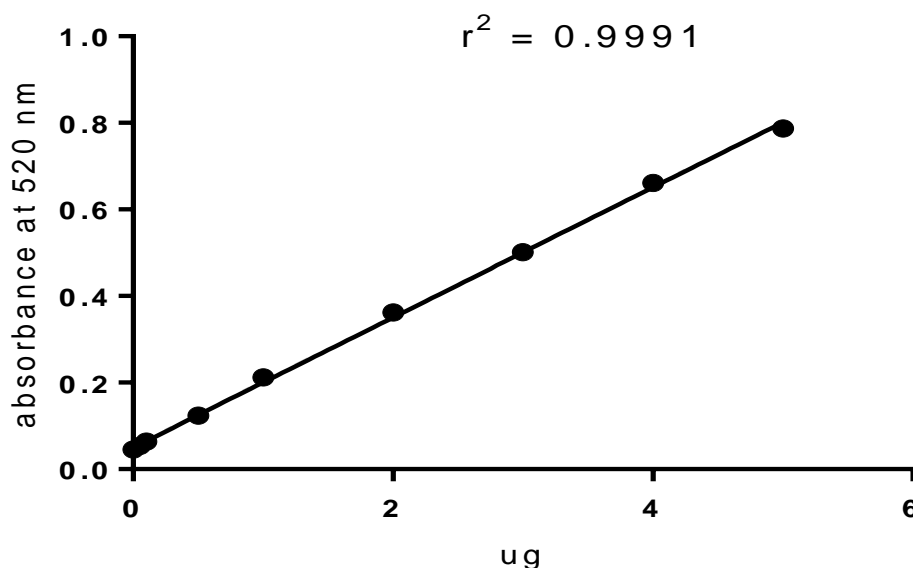


Figure 10: Standard curve for creatinine to determine the concentration of creatinine in the dried urine spots and urine

3.9.7. Determination of protein concentration in plasma and dried plasma spots

The Markwell modification method of the Lowry protein assay method was used to quantify the concentration of proteins in plasma and dried plasma spots (Markwell M.A.K et al., 1978). The Markwell modification included SDS to assist in the solubilising of proteins especially in lipoproteins and membranes. A standard curve using bovine serum albumin (BSA) was prepared, in which the mass range of protein was 1-100 μg . Two spots of filter paper containing dried plasma (equivalent to 7.4 μL plasma) were punched and placed into a 1.5 mL microcentrifuge tube. Proteins were extracted into water by adding 100 μL of dH_2O and the tube was vortexed to get good extraction. 10 μL of mixture was added to a fresh 1.5 mL microcentrifuge tube and 90 μL of dH_2O was added. 300 μL of 100:1 Markwell reagent to 4% CuSO_4 was added, the tube was incubated at room temperature for 15 minutes and 30 μL of 1:1 Folin reagent: dH_2O was added. The tubes were incubated for 45 minutes at room temperature, 300 μL of the mixture were placed into a 96-well microtiter plate and the absorbance was read at 660 nm by spectrophotometry. 7.4 μL of whole plasma was added to a separate microcentrifuge tube and treated the same as above. The protein concentration was derived from the non-linear regression of a BSA standard curve with volumes detailed in Appendix II (6).

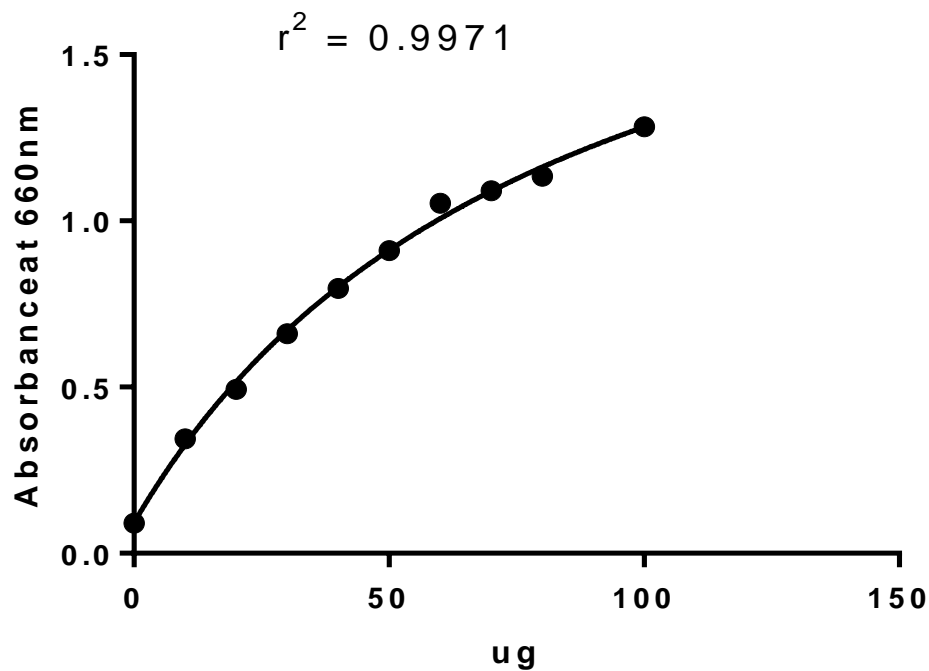


Figure 11: Standard curve for BSA to determine the protein concentration in dried plasma spots and plasma

3.9.8. Sodium Dodecyl Sulphate -Polyacrylamide Gel Electrophoresis (SDS-PAGE) of plasma spots

Electrophoresis can separate macromolecules for example protein, RNA and DNA in an electric field based on charge and size (Heller C, 1995). The SDS-PAGE method for separating protein, which uses polyacrylamide as a support medium and SDS as a means to denature the protein, is also known as the Laemmli method (Wessel D and Flugge U.I, 1984).

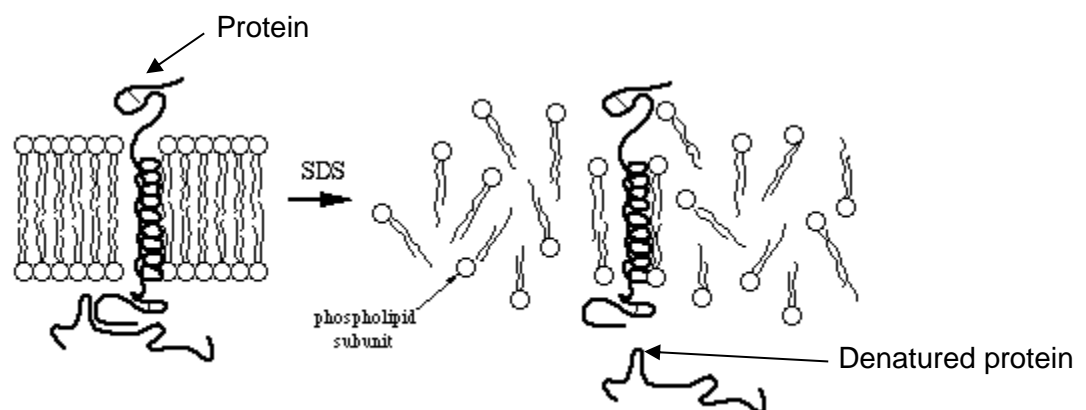


Figure 12: Illustration of protein denaturation by SDS

In SDS-PAGE the protein is in its primary structure. To achieve this denatured protein using SDS, heat is applied, and 2-mecaptoethanol is added for the reduction of sulfhydryl bonds. SDS breaks up the two and three-dimensional structure of the protein and this, combined with the negative charges, from the SDS enhances migration.

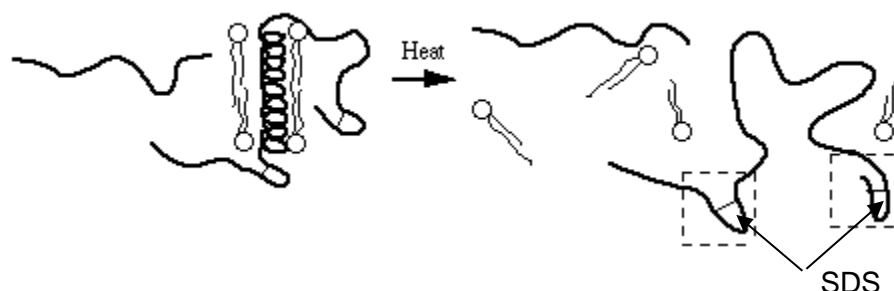


Figure 13: Graphical representation of denaturation of proteins by heat

2-mercaptoethanol breaks the disulfide bonds because it is a strong reducing agent. Heating the protein to 60 °C allows SDS to bind in the hydrophobic regions and complete the denaturation. Migration in SDS PAGE is log-linear. A gradient of 5-20 % acrylamide mass/volume separates the complete range of plasma proteins from 10 kDa to 1000 kDa. The presence of protein is demonstrated by using its affinity for Coomassie Blue stain.

3.9.8.1. Method for dried spot protein analysis by electrophoresis

In order to determine the extraction of protein from the filter paper spots, two filter paper spots containing dried plasma equivalent to 7.4 μL of whole plasma were punched and placed into each of four 1.5 mL microcentrifuge tubes. 100 μL of either water, physiological saline (0.9% NaCl), 0.1 M NaOH or 1 M NaOH were added to each tube respectively. Each tube was then heated at 60 °C for 10 minutes. 11 μL of each extract was pipetted into 4 fresh 1.5 mL microcentrifuge tubes and 14 μL of sample treatment buffer with 2 μL of saturated sucrose was added to each tube to increase the density of the solution. 10 μL of each sample was loaded onto a 5-20 % gradient polyacrylamide gel (details in Appendix II (7)), and the gel was electrophoresed for four hours at 100 volts.

The gel was removed and placed in a glass container, and Coomassie Blue stain (Appendix II (7(I))) was added. It was left for four hours at room temperature (staining hours can be reduced to 1 hour at 45 °C). Destain I (Appendix II (7)) was then used for destaining the gel for 1 hour at room temperature with gentle shaking, followed by Destain II (Appendix II (7)), which was used for 6 hours, also at room temperature with gentle shaking. After that the gel was imaged on the Gel Doc™ EZ Imager (Bio-Rad, South Africa).

3.9.9. Determination of salicylate concentration in serum and dried serum spots

Salicylate (Aspirin) poisoning is a major clinical hazard resulting from accidental ingestion in children, or suicidal overdose in adults and teenagers (Temple AR, 1981). Salicylate levels in serum are determined in dried spots using the method previously described by Trinder P, 1954. This method is based on the production of a violet coloured complex caused by the reaction between iron and salicylate.

3.9.9.1. Method for salicylate concentration determination

Two spots of filter paper containing serum (equivalent to 7.4 µL of serum) were punched out and placed in a 1.5 mL microcentrifuge tube. 100 µL of dH₂O was added and the tube was vortexed thoroughly. 50 µL of the mixture was transferred into a new tube. 50 µL of dH₂O and 300 µL of the Colour Reagent (a solution that forms a purple complex between phenols and ferric ions) was added and centrifuged at 500 x g for 2 minutes. 300 µL of the clear supernatant were transferred into a 96-well microtiter plate and the absorbance was read at 540 nm on the spectrophotometer as previously described. The salicylate concentration was derived from linear regression.

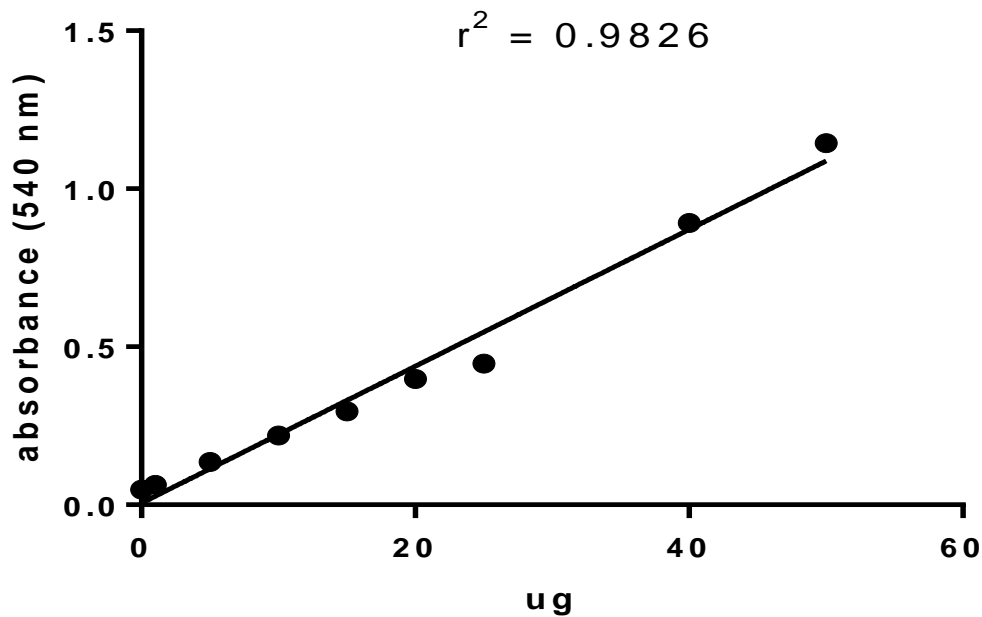


Figure 14: Standard calibration curve for salicylate to determine the concentration of salicylate in the dried serum spots and whole serum

3.9.10. Determination of cyanide concentration in whole blood and dried blood spots

Cyanide is one of the most lethal and dangerous poisons. Studies have shown that cyanide from fires burning synthetic nitrogen-containing materials may cause acute toxicity symptoms through inhalation exposure as well as from that ingested from a suicide or homicide attempt (Chin R.G and Calderon Y, 2000; Kulig W.K and Ballantyne B, 1993; Padwell A, 1997). Cyanide levels in dried blood spots were determined using the method previously described by Lundquist (Lundquist P et al. 1985). The cyanide in the sample is acidified with sulphuric acid to convert it to the volatile HCN which is trapped into sodium hydroxide and quantified by the König reaction, with sodium hypochlorite as the chlorinating agent. The cyanide concentration was derived from linear regression of a range of cyanide standards (Figure 15)

3.9.10.1. Method for cyanide concentration determination

One filter paper spot containing whole blood (equivalent to 60 μL of whole blood) was shredded into a 1.5 mL microcentrifuge tube. 300 μL of dH_2O was added, the tube was vortexed thoroughly and the mixture was transferred to a clean tube. A series of dilutions of 1 mM potassium cyanide was prepared to create a standard curve with amounts ranging from 0.5 μmol to 50 μmol . To set up the assays, the tubes containing extracted whole blood on the DBS were placed in a jar containing marbles to support the tubes. A second tube containing 200 μL of 0.1 M NaOH was placed in the same jar and 250 μL of 11 M sulphuric acid was added to the tube containing extracted whole blood. The jar was immediately sealed tightly and placed on the shaker for overnight (gentle) shaking, allowing the complete transfer of HCN to the 0.1 M NaOH. After overnight shaking the 0.1 M NaOH tube was taken out and 20 μL of 2 M acetic acid and 5 μL of 5 mM NaClO were added. The tube was vortexed. Within 1 minute, 25 μL of barbituric acid-pyridine reagent was added and the tube was vortexed. The absorbance was read within 5 to 15 minutes after addition of the barbituric acid-pyridine reagent at 580 nm on the spectrophotometer as previously described.

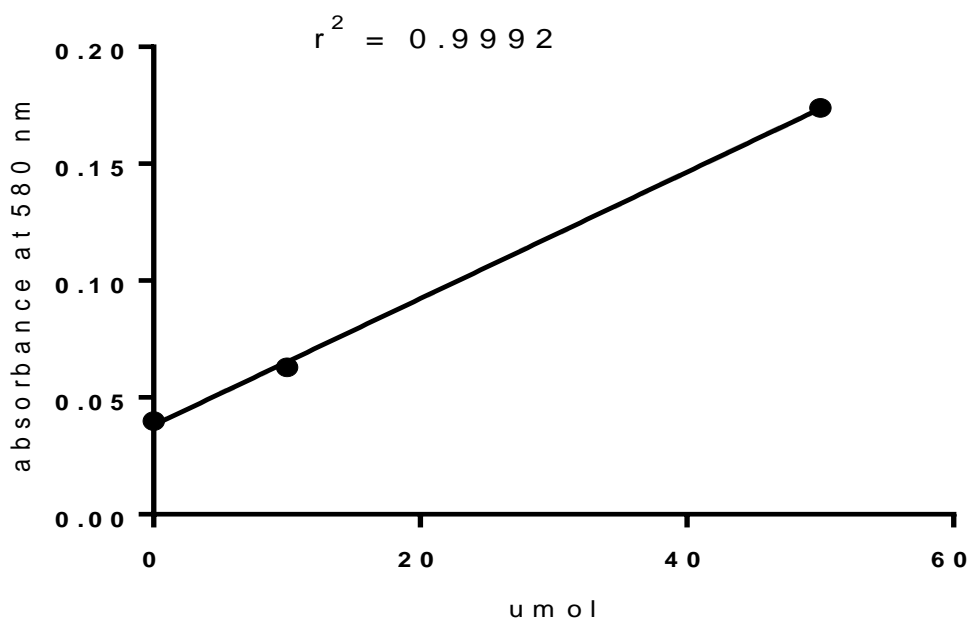


Figure 15: Standard calibration curve for cyanide to determine the concentration of cyanide in the dried blood spots and whole blood

3.9.11. Determination of thiobarbituric acid reactive substances (TBARS) concentration in serum and dried serum spots

The TBARS are formed as products of oxidation of unsaturated fatty acids in lipids and may be detected using thiobarbituric acid (TBA) as a reagent. The assay measures malondialdehyde (which is one of several TBARS and is a low molecular weight secondary product), as well as other aldehydes in the serum. One molecule of malondialdehyde (MDA) in serum condenses with two molecules of TBA reagent to give a red pigment that can be measured spectrophotometrically.

In order to measure the amount of MDA in human serum, the TBARS assay was performed according to the method of Asakawa and Matsushita (Asakawa T and Matsushita S, 1979).

3.9.11.1. Method for TBARS concentration determination

Conventionally BHT is would not be added to the sample but for this study, the BHT was added to the filter paper so that it was not required at venepuncture. The filter paper was pre-soaked with 4 mmol/L BHT/ethanol to prevent lipid peroxidation. The lipids were extracted by the procedure of Folch, Lees and Sloane Stanley (Folch et al., 1957). Three filter paper spots of dried serum (equivalent to 11.1µL of whole serum in total) were prepared by extracting into 405 µL of 2:1 chloroform: methanol, followed by microcentrifugation at 1500 x g for 10 minutes. 100 µL of dH₂O was added and the bottom organic layer was transferred to a new tube and dried under nitrogen gas. The dried lipids were dissolved in 50 µL chloroform and mixed with 50 µL of ferric chloride and 50 µL of 0.3 mmol/L BHT/ethanol and vortexed for 10 seconds. TBA reagent (0.5 g% TBA + 0.3 g% SDS) (750 µL) and glycine buffer (0.2 M glycine HCl, pH= 3.6) (750 µL) were added and the mixture was vortexed again. The reaction mixture was then incubated for 20 minutes at 100 °C in a heating block. The tubes were cooled on ice for 2 minutes to stop the reaction. After leaving the tubes at room temperature for approximately 3 minutes, 500 µL of glacial acetic acid and 1000 µL chloroform were added into the tubes. The tubes were centrifuged for 15 minutes at 1500 x g and 300 µL of the supernatant were transferred to a flat-bottom 96-well microtiter plate and the absorbance was measured at 532 nm on the spectrophotometer as previously described. 11.1 µL of whole serum was added to a separate microcentrifuge tube and treated the same as above.

The TBARS concentrations were calculated by using the molar extinction ($1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

3.9.12. Determination of conjugated dienes (CD) concentration in serum and dried serum spots

CDs are formed as a result of a re-arrangement of 2 double bonds in a polyunsaturated fatty acid during which process the methylene interruption is lost and the PUFA becomes more susceptible to lipid peroxidation. In the presence of free radicals, the hydrogen atom adjacent to the double bond is abstracted and to maintain the stability, the double bond flips to where the hydrogen atom was removed. CDs are not in a strict sense oxidation products of fatty acids, but indicate a change after free radical formation. In human serum, many CDs are accounted for by a linoleic acid isomer (octadeca-9 (cis)- 11-(trans)-dienoic acid) (Cho H et al., 2005).

3.9.12.1. Method for CD concentration determination

Three punched spots of dried serum (the equivalent of 11.1 μL) were extracted according to the method of Folch (Folch et al., 1957) using 405 μL of 2:1 chloroform: methanol, followed by centrifuging at 1500 x g for 10 minutes. The resulting monophasic was split into 2 layers by the addition of 100 μL of dH_2O and the bottom, organic layer was transferred to a new tube and dried under nitrogen gas. Chloroform has a high absorbance so the sample has to be completely dry before the addition of cyclohexane. The dried lipids were re-dissolved in 350 μL cyclohexane and 300 μL of the mixture was transferred to a 96-well microtiter UV plate and the absorbance was measured at 234 nm. The concentration was calculated using the extinction coefficient $2.95 \times 10^4 \text{ /M/cm}$. 11.1 μL of whole serum was added to a separate microcentrifuge tube and treated the same as above.

3.9.13. DNA extraction

Two filter paper spots containing buffy coat (equivalent to 100 μL whole buffy coat) were shredded and placed into a 5mL microcentrifuge tube with 1 mL physiological saline and left to stand for 10 minutes at room temperature. The tube was then vortexed for 10 seconds. The solution was transferred into a clean 1.5 mL microcentrifuge tube and centrifuged at 1000 x g

for 10 minutes. The supernatant was discarded and the cell pellet was used to perform the DNA extraction.

Genomic DNA was extracted using the QIAamp® DNA mini kit from Qiagen. 300 µL of RBC Lysis Solution was added to a 2 mL centrifuge tube. 100 µL whole blood was added and mixed by inverting. The tube was incubated for 5 minutes at room temperature (15-25°C) and was inverted gently 3 times during the incubation. After that the tube was centrifuged for 2 minutes at 2000 x g to pellet the white blood cells. The supernatant was carefully discarded, leaving the pellet in the tube. The tube was vigorously vortexed for 10 seconds to re-suspend the pellet in the residual liquid (vortexing greatly enhances cell lysis in the next step) and the pellet was completely dispersed after vortexing. 33.3 µL Protein Precipitation Solution was added to the centre of the sample, 100 µL Cell Lysis Solution was then added to the mixture which was vortexed vigorously for 20 seconds to lyse the cells and precipitate the proteins. The tube was then centrifuged for 6 minutes at 200 x g (the precipitated proteins formed a small, dense, dark brown pellet). If the protein pellet was not sufficiently compacted, it was incubated on ice for 5 minutes and the centrifugation was repeated. 100 µL isopropanol was pipetted into a clean 1.5 mL centrifuge tube and the supernatant from the previous step was added by pouring it carefully across. Care was taken that the protein pellet was not dislodged during pouring. The tube was mixed by inverting gently 50 times, and was then centrifuged for 3 minutes at 2000 x g. The DNA was visible as a small white pellet. The supernatant was carefully discarded, and the tube was drained by inverting onto a clean piece of absorbent paper for 1 minute, taking care that the pellet remained in the tube. 100 µL of 70% ethanol was then added; the tube was centrifuged for 1 minute at 2000 x g and the supernatant was discarded. The tube was drained on a clean piece of absorbent paper for 5 minutes, taking care that the pellet remained in the tube. Over-drying of the pellet was avoided, as the DNA would be difficult to dissolve. 10 µL DNA Hydration Solution was added and the mixture vortexed for 5 seconds to mix. The tube was then incubated at 65 °C for 1 hour to dissolve the DNA and then incubated at room temperature overnight with gentle shaking. The sample was then mixed gently and transferred to a storage tube.

3.9.14. Determination of DNA concentration

The DNA concentration was determined by measuring absorbances at 260 nm, 280 nm and 320 nm, using the NanoDrop 2000c Spectrophotometer (Thermo Scientific, South Africa). The ability of the nucleotides in RNA and DNA to absorb at 260 nm contributes to the total absorbance of the sample. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. A_{260/280} nm is used to determine protein contamination of a nucleic acid

sample, and ideally the ratio should be between 1.8 to 2.0. Nucleic acids absorb UV light at 260 nm due to the aromatic moieties within their structure, and proteins and phenolic compounds have strong absorbance at 280 nm with the aromatic amino acid (tryptophan, phenylalanine, tyrosine and histidine) being responsible for absorbance (Holden M.J. et al., 2009 and Glasel, J. A, 1995).

To further check the DNA purity, the absorbance at 320 nm was measured to detect other possible contaminating compounds.

$$\text{DNA purity (A260/A280)} = (\text{A260} - \text{A320}) / (\text{A280} - \text{A320})$$

The DNA concentration was determined by measuring absorbance at 260 nm, adjusting the A260 measurement for turbidity (measured by absorbance at 320 nm), then multiplying by the dilution factor, and using the relationship that an absorbance at A260 of 1.0 = 50 µg/mL pure dsDNA

$$\text{The DNA Concentration (}\mu\text{g/mL)} = (\text{A260} - \text{A320}) \times \text{dilution factor} \times 50 \mu\text{g/mL}$$

3.9.15. Polymerase Chain Reaction (PCR)

To verify the quality of extracted genomic DNA, screening for mutations in exon 2 of the *STAP* gene was carried out using quantitative real time PCR high-resolution melting (RT-qPCR-HRM). The extracted genomic DNA was amplified on the LightScanner®32 (Idaho Technology Inc.) and HRM-enabled real time PCR machine. The PCR reaction mixture contained 3.3 µL of water, 1 µL of 10 X buffer (10 X (20 nM Mg₂Cl)), 1 µL of 2 mmol/L dNTP's, 1 µL of 5 µmol/L forward primer, 1 µL of 5 µmol/L of reverse primer, 0.7 µL of LC green dye, 1 µL of TSG Taq and 1 µL of 1 µg/µL extracted DNA. The amplification step was carried out according to the following PCR program for *STAP* gene: initial denaturation of 10 minutes at 95 °C; followed by 50 cycles of 95 °C for 10 seconds, 63 °C for 20 seconds, and 72 °C for 20 seconds, concluding with 7 minutes at 72 °C and 1 minute at 4 °C. The PCR products were analysed by the HRM program to produce a derivative curve for easy analysis. Sequence variation was screened by curve analysis in a single run.

3.9.16. Statistical analysis

Conventional descriptive statistics was used to describe the data with the use of GraphPad Prism 6 software (GraphPad Software Inc., San Diego, USA) using mean, SD, quartile, median, and 95 % confidence intervals. Normally distributed variables were expressed as mean \pm standard deviation and nonparametric variables were expressed as median and interquartile range. Comparisons were carried out with the software according to the distribution of the data and whether data were paired or fitted repeated measures. Bland-Altman plots were also carried out using Graphpad Prism version 6. A p value < 0.05 was considered statistically significant.

Chemical reagents

Unless stated in the text, all reagents were supplied by Sigma-Aldrich, Munich, Germany.

4. RESULTS

4.1. Spotting

4.1.1. Surface area of increasing volumes of biologic fluids spotted onto filter paper

To determine the surface area of each spot in relation to volume for various biologic fluids (whole blood, plasma, serum, and urine) each was spotted on the filter paper, left to dry at ambient temperature for 3 hours and the surface area was calculated using a transparent paper graph sheet marked with 2 mm squares (Section 3.6.2). This enables rapid and reproducible calculation of the spot surface areas. Most urine spots after drying kept the original colour of the filter paper, but some did not, depending on the original appearance of the urine. To visualise the outline of the dried urine spot, ultraviolet light was used to show the blue edge, characteristic of biologic fluids (Stevens D.S and Dick G.E.F, 1946) and those borders were then marked with pencil. Using a transparent graph sheet marked with 2 mm squares allowed the calculation of the spot surface areas. Using linear regression analysis, the relationship between spot surface area (y) and volume (x) applied on the filter paper was calculated. This resulting equation described the behaviour of the volume of each biologic fluid on the filter paper spot.

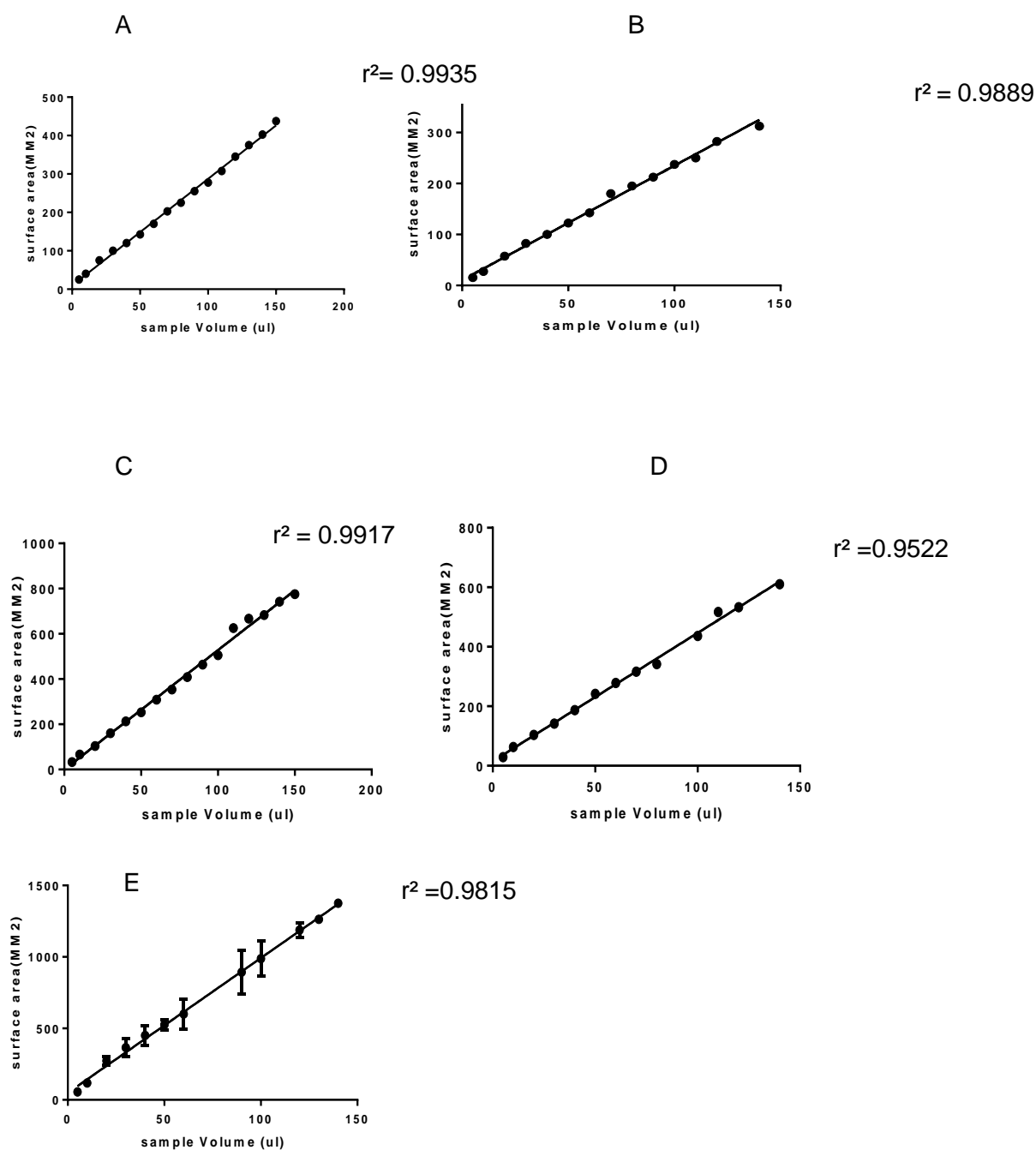


Figure 16: Correlation of surface areas of biologic fluids spotted on the filter paper with the volume of the spot (A) whole blood, (B) buffy coat, (C) plasma, (D) serum and (E) urine

4.1.2. The effect of haematocrit on spotting of whole blood onto filter paper

To determine the effect of haematocrit on the spread of whole blood on the filter paper, the whole blood was spun to separate cells from plasma. Various volumes of plasma were then used to create a wide range of haematocrit. Figure 17 shows that there were no differences in the spread of the whole blood on the filter paper from 25 % to 71 %. Below 25 % haematocrit, the spot became bigger and above 71.4 % haematocrit there was less spread of whole blood on the filter paper. While the graph shows the behaviour is sigmoidal, the range 25-70 % is for practical purposes suitable for spotting. Only extreme cases of anaemia may spread to larger surface area. Haematocrit is not a significant influence on preparation of blood for spotting, haematocrit should be noted as the ratio of cell height to total height at venepuncture in the collection tube to indicate whether the sample is suitable for deriving its volume by surface area.

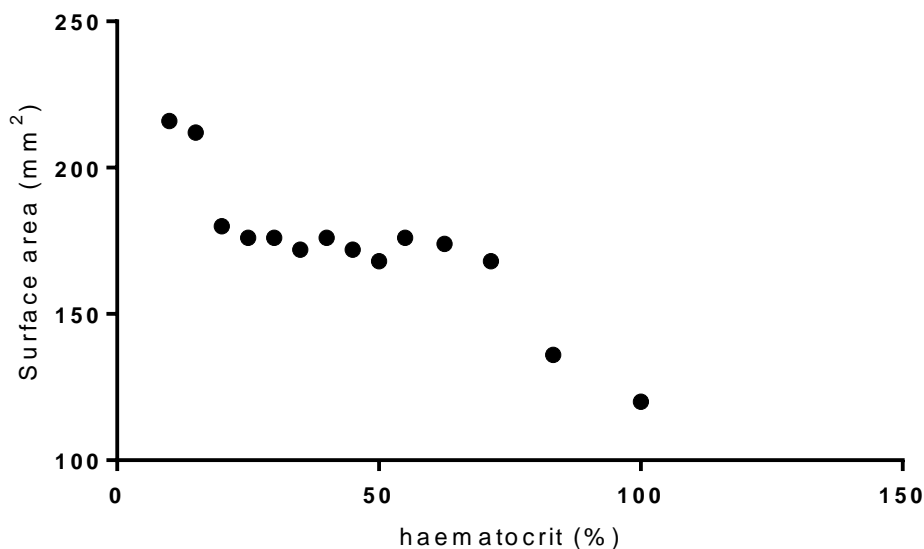


Figure 17: Graphical representation of the effect of haematocrit on the spread of blood on the filter paper

4.1.3. The sealing of filter paper

All 6 sealed filter paper samples with cobalt chloride turned pink after 8 days at ambient temperature (Section 3.8), indicating that the plastic seal did not work optimally and is permeable to water vapour. Heated cobalt chloride impregnated silica crystals were placed with filter paper into a 15 mL centrifuge tube, the cap was closed and wrapped with parafilm "M" Laboratory film (American National Can, Greenwich, CT, USA) to make sure air did not enter. The cobalt chloride also turned pink after 8 days, indicating the exposure of water vapour to cobalt chloride, leading to a change in colour of the crystals. This indicates that exposure to air could provide adequate water vapour to allow the hydration of cobalt chloride.

To confirm these results, an alternative indicator of hydration, copper sulphate, was employed. Copper sulphate was heated for 4 hours at 90 °C to remove any water. Cards were then vacuum sealed with this anhydrous copper sulphate along with filter paper samples under various conditions: 2 pouches were immersed in water, another 2 left at room temperature and another 2 placed at -20 °C, Figure 18. Each was checked daily for any colour change. The copper sulphate with filter paper did not turn blue under water, at ambient temperature or at -20 °C for 3 weeks. However, the copper sulphate and filter paper immersed under water turned blue after 25 days and it also changed colour to a faint blue at ambient temperature, but at -20 °C remained white until 10 months.

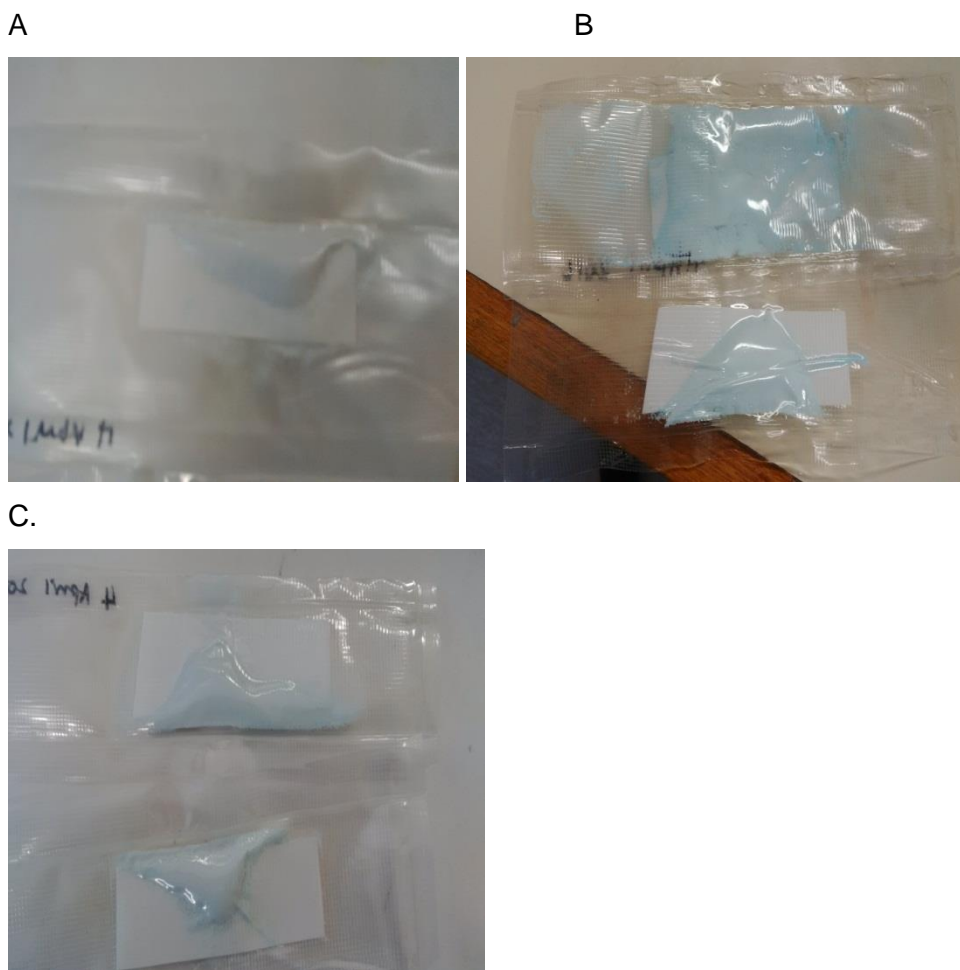


Figure 18: Photographs of copper sulphate with filter paper, immersed in dH_2O stored at room temperature, and at -20°C . A=pouches just after sealing, B=pouches after 25 days of immersion and C= pouches placed at room temperature

The sealed water (mass in g, surface area of 151.5 cm^2) at ambient temperature showed a loss of mass of 12.7 mg , indicating a loss per cm^2 of 0.08 mg . At 37°C , the loss was 81.6 mg over 129.5 cm^2 , which indicated a loss per cm^2 of 0.63 mg . Thus the plastic sealer is permeable at 37°C but very little at ambient temperature.

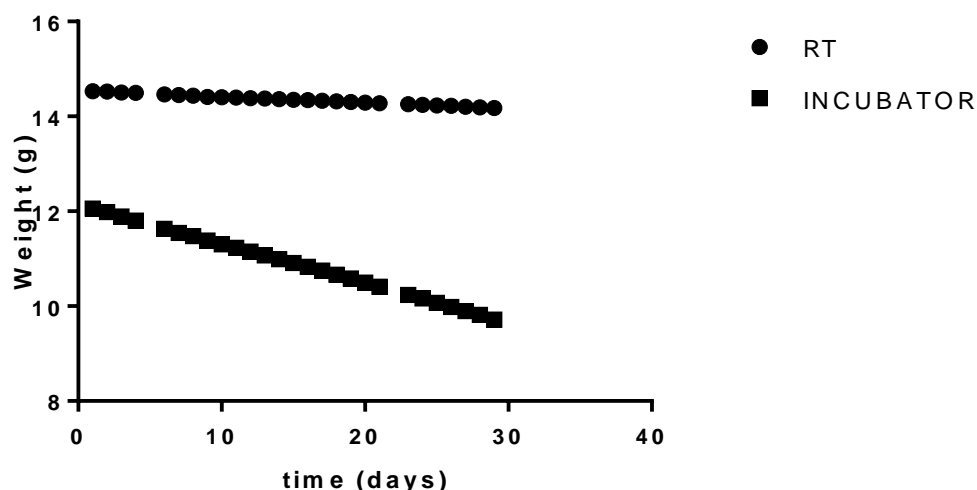


Figure 19: A graphical representation of the rate at which the sealed water lost mass (g) per day stored at 2 different temperatures. RT for sealed ice cubes that were stored at room temperature; INCUBATOR for sealed ice cubes that were kept at 37 °C in an incubator

4.2. Concentration of cholesterol in dried plasma spots and in whole plasma

To determine if the DPS can be used for diagnosis of lipid disorders, the plasma from 14 volunteers was spotted on the filter paper and dried and analysed after 1 week, 3 months and 7 months of storage at room temperature, 4 °C and – 20 °C both sealed and unsealed. This applied to the analysis of the cholesterol, triglycerides and phospholipids. The original plasma from the volunteers was aliquoted and stored at -20 °C and analysed together with the DPS. The whole plasma was aliquoted into 1.5 mL microcentrifuge tubes (to avoid freeze-thawing of the plasma) and stored at -20 °C.

The concentration of cholesterol in the DPS ranged between 2.77 and 5.97 mmol/L and in the whole plasma ranged between 3.30 and 7.58 mmol/L. There were no significant differences in the original samples compared to samples analysed after 1 week, 3 months and 7 months. There was a significant difference (paired non-parametric Anova, $p < 0.001$) between the sample after 1 week after collection and three months of storage at room temperature in both sealed and unsealed, sealed at 4 °C and at -20 °C compared to whole plasma sample. There were no significant differences between the DPS stored under different conditions. The relationship between the whole plasma and DPS stored at ambient temperature sealed and unsealed, 4 °C and -20 °C were linearly and correlated with DPS stored at ambient

temperature unsealed, having $r^2 = 0.6355$, sealed at ambient temperature $r^2 = 0.8083$, stored at 4 °C with $r^2 = 0.6981$ and at – 20 °C with $r^2 = 0.6002$ respectively after a week of storage (as shown in figure 21). The concentrations of cholesterol in DPS stored at ambient temperature sealed and unsealed, stored at 4°C and -20°C were approximately 20% less than from whole plasma. The intra-assay and inter-assay coefficients of variation of the DPS were 7.8% and 14.3% respectively, and for whole plasma were 6.0% and 13.3% respectively. Figure 42 portrays the Bland-Altman plots for cholesterol analyses. This shows a bias of -0.1266 ± 0.6529 for sealed blots, -0.1811 ± 0.4848 for unsealed blots, 0.1033 ± 0.5917 for sealed blots stored at 4 °C on the filter paper blots, and similarly the 95 % limits of agreement ranged from -1.406 to 1.153, -1.131 to 0.7691, -1.057 to 1.263 respectively after 1 week of storage. One dried plasma spot stored at 4°C was outside the 3 SD limits (Figure 42). The recoveries of cholesterol from DPS that were stored at ambient temperature sealed and unsealed and stored at 4 °C and -20 °C were $78 \pm 12.2 \%$, $77 \pm 7.5 \%$, $82 \pm 13.4 \%$, and $81 \pm 11.4 \%$, respectively after a week of storage. It is convenient to use the dried plasma spot to determine the cholesterol concentration on the filter paper, however the sample will need to be corrected for concentration as in the whole plasma. More detailed studies are indicated to determine the reason for incomplete recovery, for possible improvements of recovery and the derivation of a correction factor.

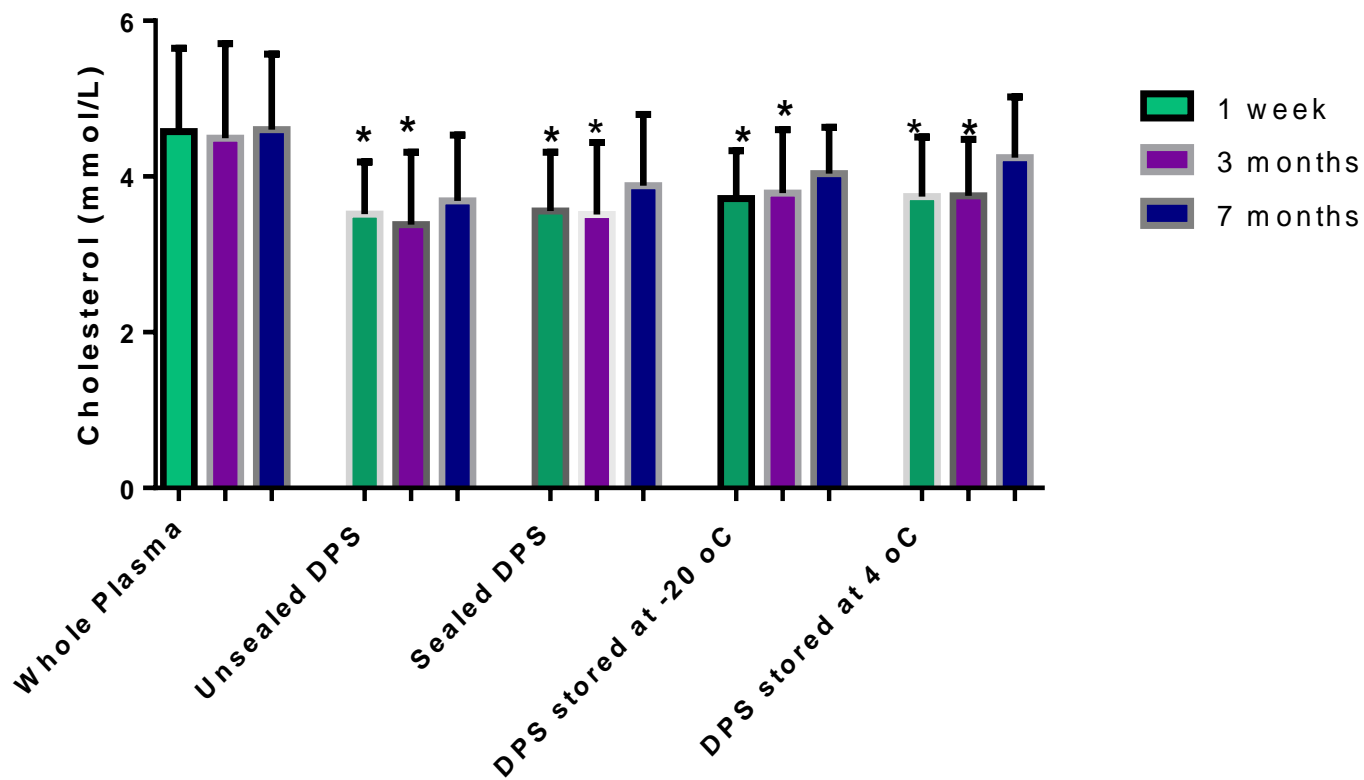


Figure 20: Comparison between cholesterol concentration in whole plasma and DPS samples stored at different temperatures and analysed after 1 week, 3 months and 7 months of spotting. Green for samples analysed after 1 week of storage, purple for samples analysed after 3 months of storage and blue for samples analysed after 7 months of storage , * $p < 0.05$. Each of the spot samples was compared to the original samples for statistical analysis. The data was non-parametric

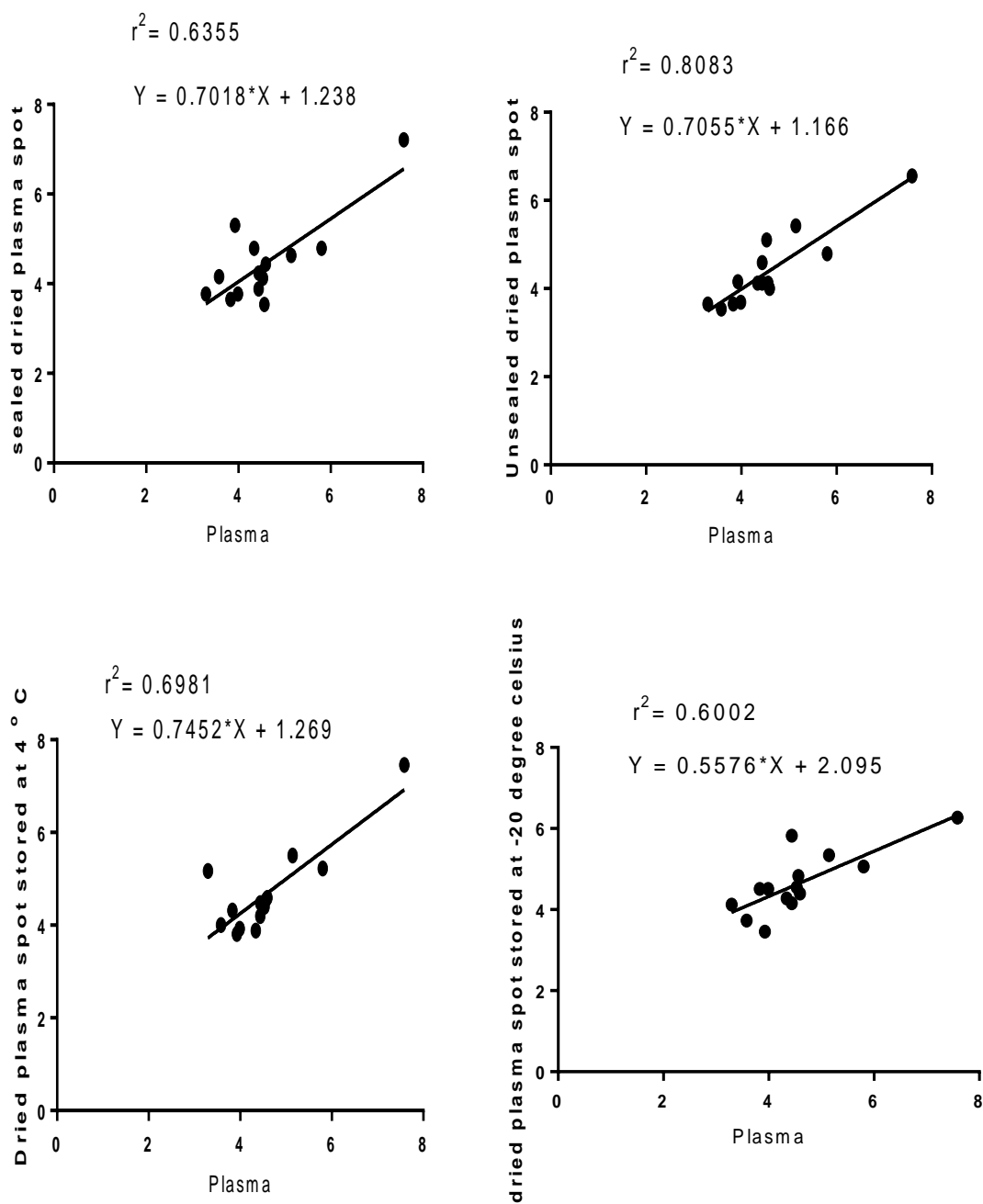


Figure 21: Correlation between cholesterol concentration in whole plasma and DPS samples stored at ambient temperature, 4 °C and -20 °C after a week of storage

4.3. Concentration of triglyceride in dried plasma spots and in whole plasma

The triglyceride concentration was determined using enzymatic assays for whole plasma and DPS that were stored at ambient temperature both sealed and unsealed, sealed at 4 °C and -20 °C and analysed after blood collection stored for 1 week, 3 months and 7 months. The whole plasma was aliquoted into 1.5 mL Microcentrifuge tubes (to avoid freeze-thawing of the plasma) and stored at -20 °C.

The median (interquartile range) for triglyceride concentrations in the DPS were 1.48 (1.253 – 1.824) mmol/L, 1.463 (1.332 – 1.890) mmol/L, 1.605 (1.315 – 1.838) and 1.671 (1.293 – 1.912) mmol/L stored unsealed, sealed at room temperature, 4 °C and -20 °C sealed respectively and that of the corresponding whole plasma was 1.653 (1.239 – 1.883) mmol/L after 1 week of storage.

There were no significant differences (paired non-parametric Anova, $p > 0.05$) between the DPS and whole plasma. The relationship between the fresh plasma and DPS stored at ambient temperature sealed and unsealed, 4 °C and -20 °C were linear and correlated after 1 week of collection, with r^2 values of 0.6366, 0.7192, 0.5786, and 0.5069 (Figure 23) after a week of collection. The recoveries of triglyceride from DPS that were stored at ambient temperature sealed and unsealed and stored at 4 °C and -20 °C were 97 ± 9.02 %, 97 ± 13.47 %, 106 ± 10.00 %, and 97 ± 9.07 %, respectively after a week of storage. The intra-assay and inter-assay coefficients of variation of the modified method for DPS were 6.0 % and 7.6 % respectively, and for plasma were 5.7 % and 7.7 % respectively. Figure 43 portrays the Bland-Altman plots for triglyceride analyses. This shows a bias of 0.02025 ± 0.2403 for sealed blots, 0.04995 ± 0.2648 for unsealed blots, 0.0228 ± 0.2908 for sealed blots stored at 4 °C and 0.09778 ± 0.3311 for sealed blots stored at -20 °C on the filter paper blots and similarly the 95 % limits of agreement ranged from -0.4508 to 0.4913, -0.4690 to 0.5689, -0.5478 to 0.5923 and -0.5512 to 0.7468 respectively after 1 week of storage. It is convenient to use the DPS for analysis of triglyceride concentration irrespective of the condition of DPS storage. The DPS is reliable for determination of triglyceride concentration compared to whole plasma which shows the unstable after being stored for 3 months at -20 °C.

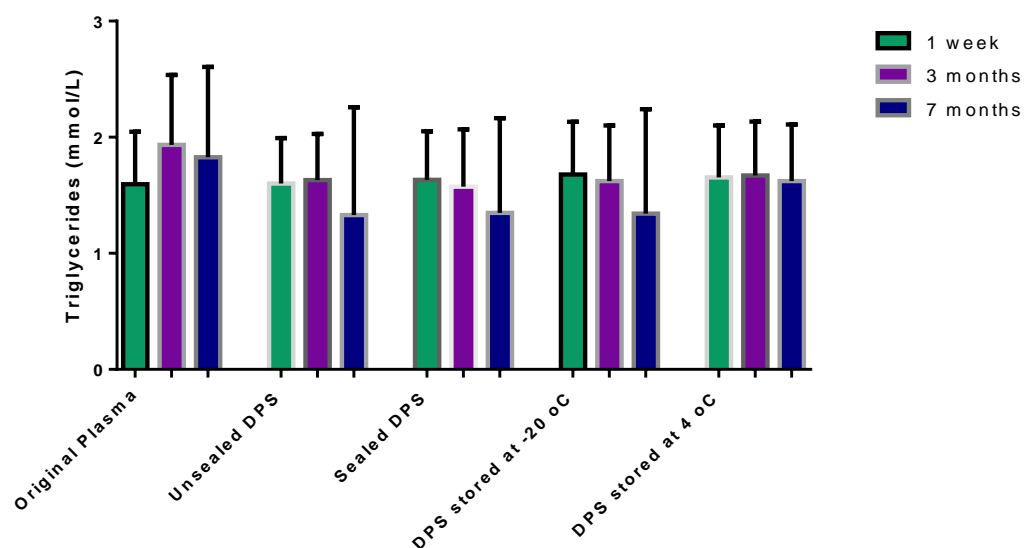


Figure 22: Comparison between triglyceride concentration in whole plasma and DPS samples stored at different temperatures and analysed 1 week, 3 months and 7 months after spotting. Green for samples analysed after 1 week of storage, purple for samples analysed after 3 months of storage and blue for samples analysed after 7 months of storage. Each of the spot samples was compared to the original samples for statistical analysis.

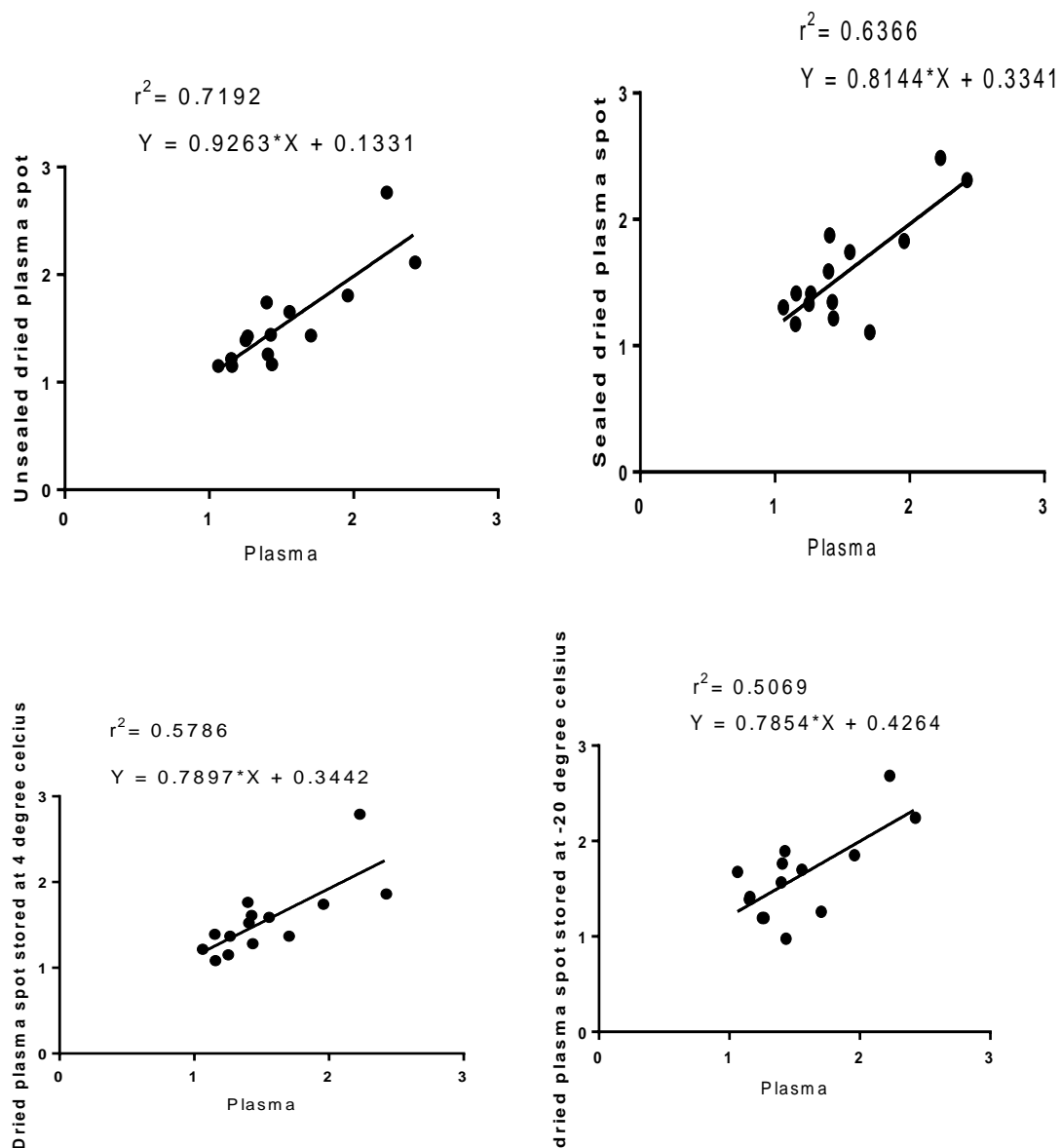


Figure 23: Correlation between triglyceride concentration in whole plasma and DPS samples stored at ambient temperature, 4°C and -20°C after a week of storage

4.4. Concentration of phospholipids in dried plasma spots and in whole plasma

The phospholipid concentration was determined using the Choline Oxidase method in the plasma and DPS samples stored, sealed and unsealed, at ambient, 4 °C and -20 °C and analysed after 1 week, 3 months and 7 months of storage. The whole plasma was sub-aliquoted into 1.5 mL Microcentrifuge tubes (to avoid freeze and thawing of the plasma) and stored at -20 °C.

The concentration of phospholipids in the DPS ranged from 0.106 to 0.724 nmol/L and in whole plasma ranged from 0.193 to 672 nmol/L respectively. There were no significant differences (paired non-parametric Anova, $p > 0.05$) between the DPS stored at different temperatures and whole plasma after 1 week of storage. However, after 3 months and 7 months there were significant differences (paired non-parametric Anova, $p < 0.0001$) between all DPS stored at different temperatures compared to whole plasma. The relationships between the whole plasma and DPS stored at ambient temperature sealed and unsealed, 4 °C and -20 °C were linear and correlated, with r^2 values of 0.5059, 0.5934, 0.4460, 0.5427, respectively after a week of storage.

The recoveries (compared to whole plasma) of the phospholipid from DPS stored at ambient temperature sealed and unsealed and stored at 4 °C and -20 °C were 104.3 ± 6.54 %, 109.4 ± 10.67 %, 108.7 ± 8.15 %, 113.5 ± 7.16 %, respectively. The intra-assay and inter-assay coefficients of variation of the modified method for DPS were 13.2 % and 11.4 % respectively, and for plasma were 13.6 % and 11.7 % respectively. Figure 44 portrays the Bland-Altman plots for phospholipid analyses. This shows a bias of 0.03971 ± 0.06024 for sealed blots, 0.04004 ± 0.06626 for unsealed blots, 0.05472 ± 0.07419 for sealed blots stored at 4 °C and 0.07448 ± 0.06383 for sealed blots stored at -20 °C and similarly the 95 % limits of agreement ranged from -0.07836 to 0.1578, -0.08983 to 0.1699, -0.09069 to 0.2001 and -0.05062 to 0.1996 respectively after 1 week of storage. The DPS stored at room temperature, both sealed and unsealed yielded determination of phospholipid concentration comparable to whole plasma. Sealed spots, stored at 4 °C and -20 °C, showed an apparent increase in concentration after 3 months of storage.

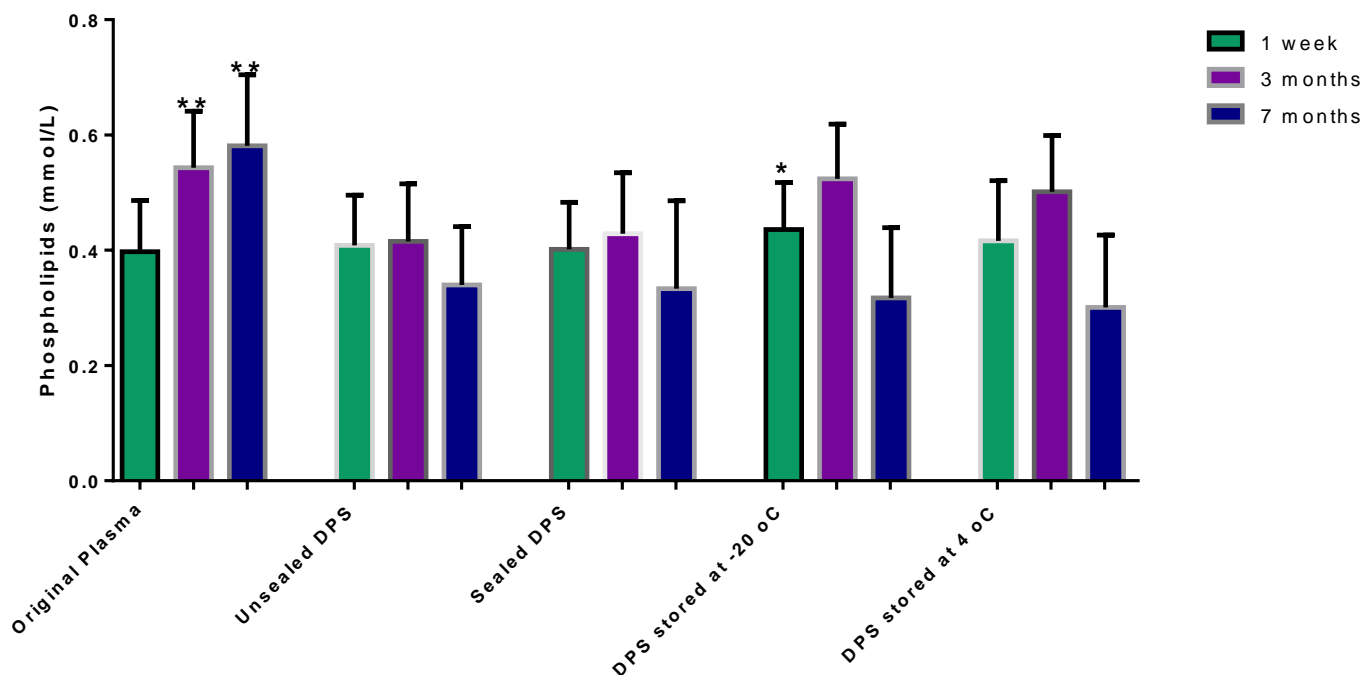


Figure 24: Comparison of phospholipid concentration in whole plasma and DPS samples stored at different temperatures analysed 1 week, 3 months and 7 months of spotting. Green for samples analysed after 1 week of storage, purple for samples analysed 3 months of storage and blue for samples analysed after 7 months of storage. * $p<0.05$ and ** $p<0.0001$. Each of the spot samples was compared to the original samples for statistical analysis.

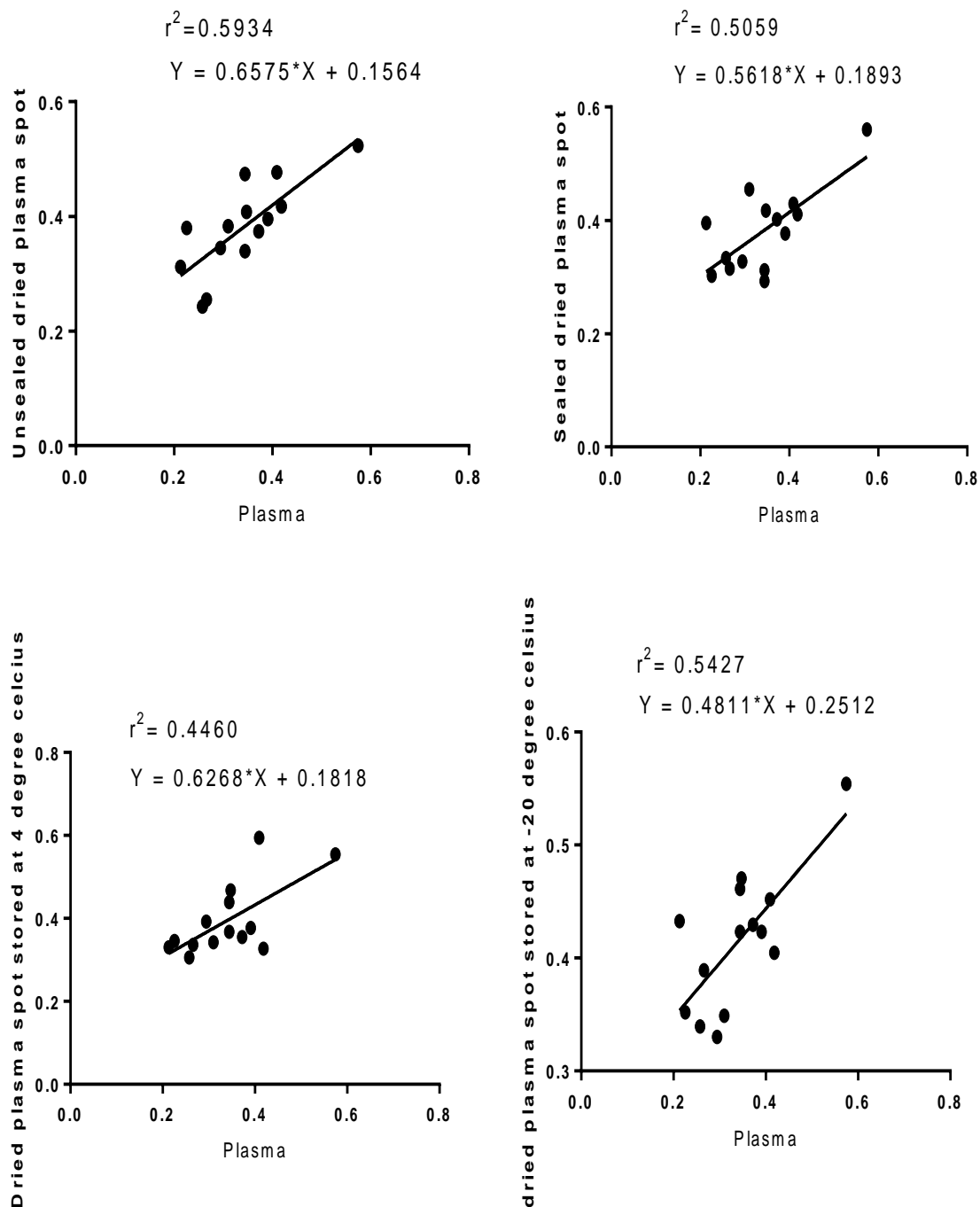


Figure 25: Correlation between phospholipid concentration in whole plasma and DPS samples stored at ambient temperature, 4 °C and -20 °C after a week of storage

4.5. Concentration of glucose in dried plasma spots and in whole plasma

To determine if the DPS can be used for diagnosis of glucose disorders (hyperglycaemia and hypoglycaemia), plasma samples from 14 volunteers were spotted on the filter paper, dried and analysed after 1 week, 3 months and 7 months of storage at room temperature both sealed and unsealed and sealed at 4 °C and – 20 °C for glucose. The glucose concentration was determined using the Mutarotase-GOD method in plasma DPS that were stored at room temperature both sealed and unsealed at 4 °C and -20 °C for 1 week, 3 months and 7 months. The whole plasma was aliquoted into 1.5 mL microcentrifuge tubes (to avoid freeze-thawing of the plasma) and stored at -20 °C. The median (interquartile range) of glucose concentrations in the unsealed and sealed stored at room temperature, sealed stored at 4 °C and -20 °C DPS and the corresponding whole plasma were 3.097 (2.724 – 4.457) mmol/L, 3.890 (3.433 – 4.343) mmol/L, 3.867 (3.412 – 4.467) mmol/L, 3.930 (3.198 – 4.416) mmol/L and 2.733 (2.359 – 3.639) mmol/L after 1 week of storage respectively. There were significant differences (paired non-parametric Anova, $p < 0.0001$) between whole plasma and sealed stored DPS at room temperature, 4 °C and -20 °C after 1 week of storage. After 7 months the means of DPS were similar to whole plasma (paired non-parametric Anova, $p > 0.05$). The relationship between the fresh plasma and DPS stored at room temperature sealed and unsealed, 4 °C and -20 °C was linear and correlated, with r^2 values of 0.8488, 0.7856, 0.7857 and 0.8828 respectively after a week of storage.

The recoveries of glucose from DPS (compared to whole plasma), that were stored at room temperature sealed and unsealed, 4 °C and -20 °C were $104 \pm 6.09 \%$, $102 \pm 6.70\%$, $103 \pm 6.20 \%$ and $112 \pm 7.94 \%$ respectively 1 week after blood collection. The intra-assay and inter-assay coefficients of variation of the DPS were 6.3 % and 8.7 % respectively and for plasma were 8.0 % and 6.7 % respectively. Figure 45 portrays the Bland-Altman plots for glucose analyses. This shows a bias of 0.8711 ± 0.6091 for sealed blots, 0.3283 ± 0.6988 for unsealed blots, 0.7542 ± 0.8107 for sealed blots stored at 4 °C and 0.8706 ± 0.6219 for sealed blots stored at -20 °C and similarly the 95 % limits of agreement ranged from -0.3227 to 2.065, -1.041 to 1.698, -0.8347 to 2.343 and -0.3484 to 2.090 respectively after 1 week of storage. The DPS is reliable for determining the glucose concentration on the unsealed spot stored for 7 months at room temperature.

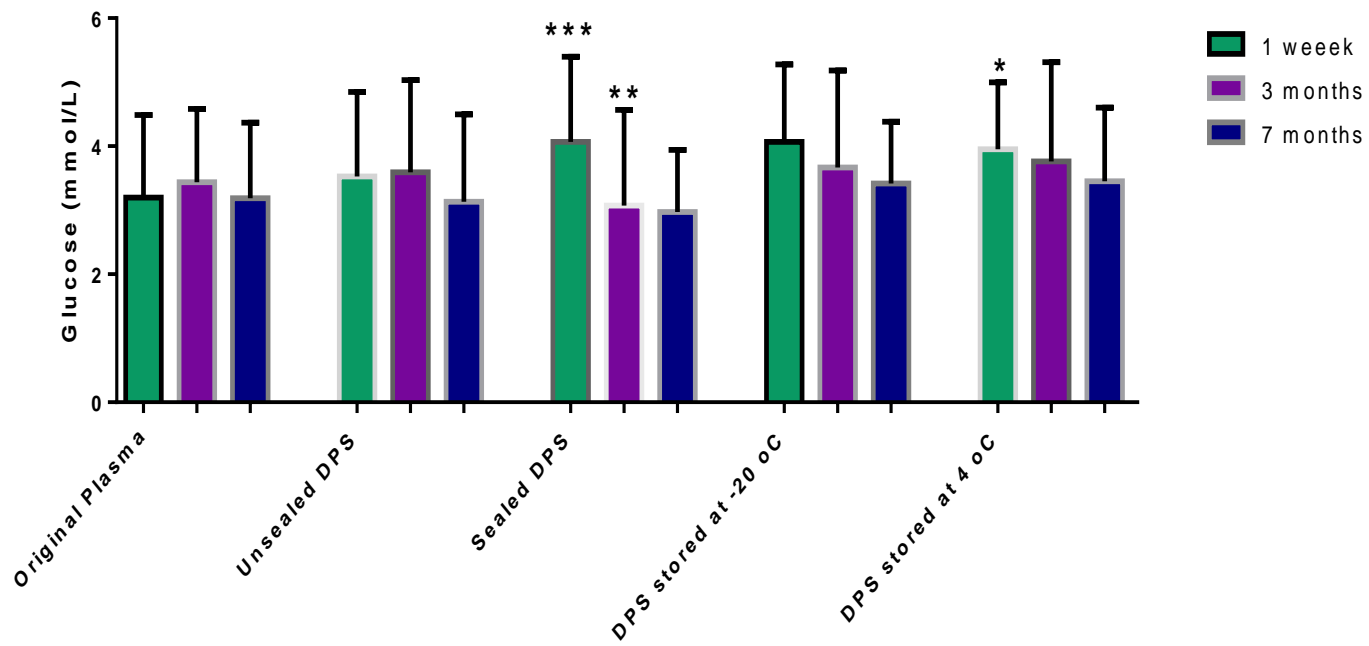


Figure 26: Comparison of glucose concentration in whole plasma and DPS samples stored at different temperatures and analysed 1 week, 3 months and 7 months after spotting. Green for samples analysed after 1 week of storage, purple for samples analysed after 3 months of storage and blue for samples analysed after 7 months of storage. * $p<0.05$, ** $p<0.001$ and *** $p<0.0001$. Each of the spot samples was compared to the original samples for statistical analysis.

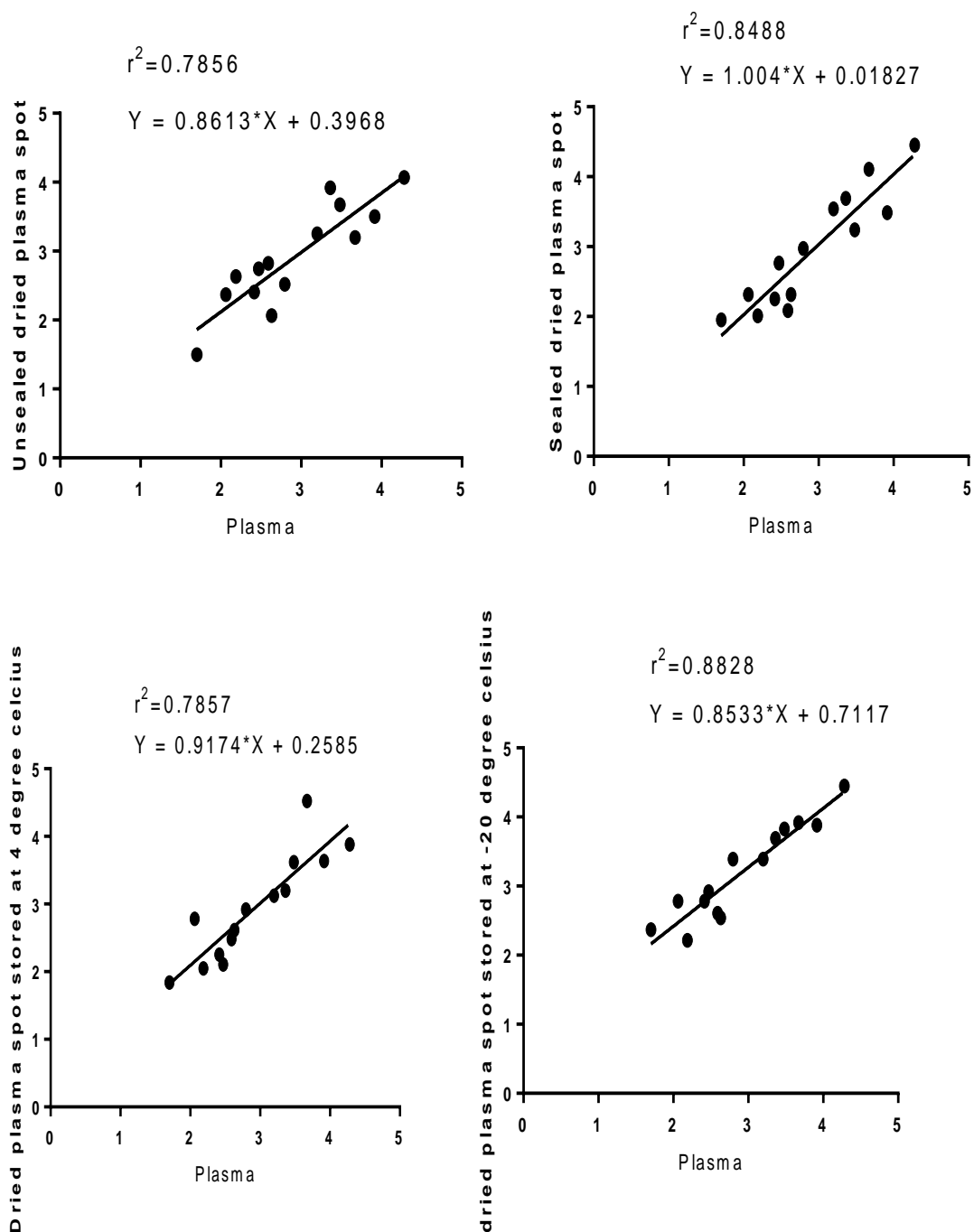


Figure 27: Correlation between glucose concentration in whole plasma and DPS samples stored at ambient temperature, 4 °C and -20 °C after a week of storage

4.6. Concentration of lactate in dried plasma spots and in whole plasma

To determine if the DPS can be used for the determination of lactate concentration, the plasma from 14 volunteers was spotted on the filter paper, dried and analysed after 1 week, 3 months and 7 months of storage at room temperature both sealed and unsealed and sealed at 4 °C and -20 °C. The lactate concentration was determined using an enzymatic kit (purchased from Randox) spectrophotometric method in plasma and DPS. The whole plasma was aliquoted into 1.5 mL microcentrifuge tubes (to avoid freeze-thawing of the plasma) and stored at -20 °C. The concentration of lactate in DPS ranged between 1.63 and 4.78 nmol/L and in whole plasma ranged between 1.40 and 3.87 nmol/L. The means of lactate were significantly different (paired non-parametric Anova, $p < 0.05$) between the DPS and whole plasma for 7 months of storage but the means were similar (paired non-parametric Anova, $p > 0.05$) compared to each group analysed at different time points other than 7 months. The relationship between the whole plasma and DPS stored at ambient temperature sealed and unsealed, 4 °C and -20 °C were linear and highly correlated, with r^2 values of 0.9567, 0.9171, 0.9458 and 0.9630, respectively after a week of storage.

The recoveries of lactate from DPS that were stored at ambient temperature sealed and unsealed and stored at 4 °C and -20 °C were $116 \pm 10.03\%$, $116 \pm 4.64\%$, $113 \pm 2.29\%$, and $113 \pm 2.25\%$, respectively. The intra-assay and inter-assay coefficients of variation of the modified method for the DPS were 4.4 % and 4.8 % respectively, and for plasma were 6.8 % and 6.0 %, respectively. Figure 46 portrays the Bland-Altman plots for lactate analyses. This shows a bias of 0.09613 ± 0.2320 for sealed blots, 0.1007 ± 0.1746 for unsealed blots, 0.08829 ± 0.1330 for sealed blots stored at 4 °C and 0.1261 ± 0.2564 for sealed blots stored at -20 °C and similarly the 95 % limits of agreement ranged from -0.3585 to 0.5508, -0.2416 to 0.4429, -0.1724 to 0.3490 and -0.3765 to 0.6287 respectively after 1 week of storage. The DPS is reliable for determination of lactate concentration in the sealed DPS stored at room temperature, 4 °C and -20 °C for 7 months with a good correlation between the whole plasma and DPS.

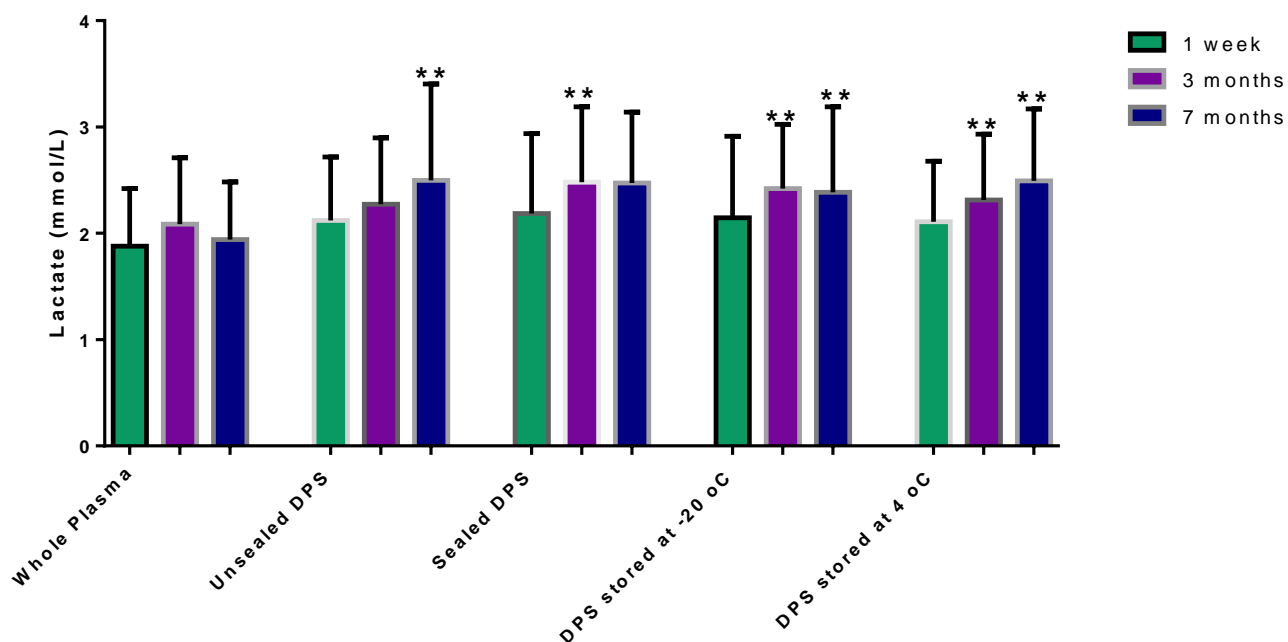


Figure 28: Comparison between lactate concentration in whole plasma and DPS samples stored at different temperatures and analysed 1 week, 3 months and 7 months after spotting. Green for samples analysed after 1 week of storage, purple for samples analysed after 3 months of storage and blue for samples analysed after 7 months of storage. * $p < 0.05$ and ** $p < 0.001$ (highly significant). Each of the spot samples was compared to the original samples for statistical analysis.

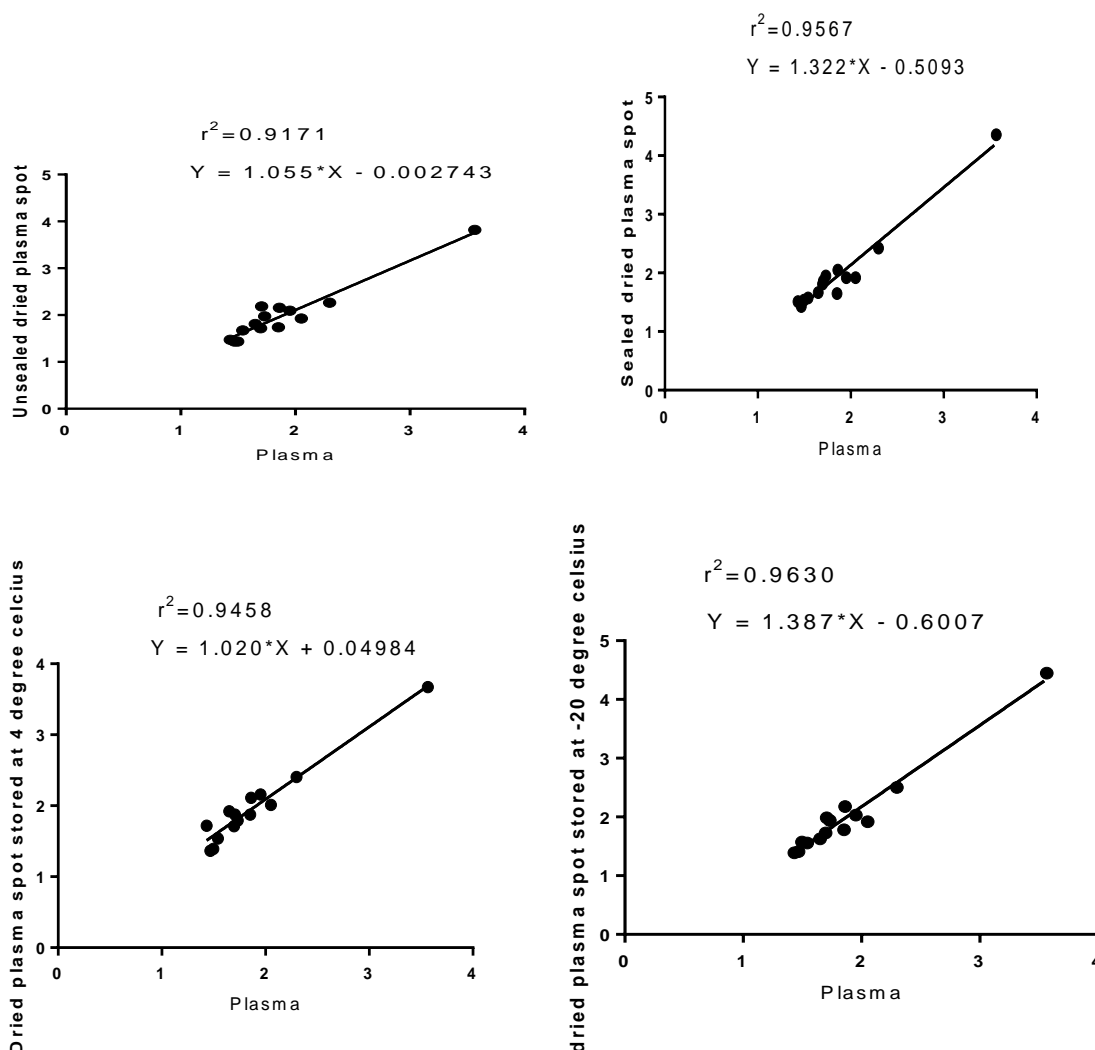


Figure 29: Correlation between lactate concentration in whole plasma and DPS samples stored at ambient temperature, 4 °C and -20 °C after a week of storage

4.7. Concentration of creatinine in dried urine spots and in whole urine

To determine if the DUS can be used for diagnosis of kidney disease, the urine from 14 volunteers was spotted on filter paper and dried and analysed after 1 week, 3 months and 7 months of storage at room temperature both sealed and unsealed and sealed at 4 °C and – 20 °C for creatinine. Creatinine concentration in urine was determined using the Jaffe method in the whole urine and DUS at ambient temperature both sealed and unsealed, 4 °C and -20 °C and analysed after collection. The whole urine was aliquoted into 1.5 mL microcentrifuge tubes (to avoid freeze-thawing of the urine) and stored at -20 °C.

The creatinine in the DUS ranged from 1.12 mmol/L to 26.7 mmol/L and in whole urine ranged from 1.60 mmol/L to 22.67 mmol/L. There were significant differences (paired non-parametric Anova, $p < 0.005$) between sealed DUS stored at room temperature, 4 °C and -20 °C compared to whole urine. The creatinine in the urine was significantly decreased (paired non-parametric Anova, $p = 0.001$) after 7 months compared to 1 week of analyses. The whole urine creatinine was significantly decreased after 7 months of storage compared to 1 week of storage (paired non-parametric Anova, $p < 0.05$) The relationships between the whole urine and DUS stored at ambient temperature sealed and unsealed, 4 °C and -20 °C were linear and highly correlated, with r^2 values of 0.9769, 0.9759, 0.9789 and 0.9719, respectively after a week of storage.

The recoveries of creatinine in urine and DUS stored at ambient temperature sealed and unsealed, 4 °C and -20 °C were 112 ± 6.01 %, 108 ± 5.97 %, 113 ± 6.31 % and 117 ± 5.74 %, respectively after 1 week of storage. The intra-assay and inter-assay coefficients of variation of the modified method for DUS were 11.0 % and 10.5 % and for urine were 11.8 % and 17.0 %, respectively. Figure 47 portrays the Bland-Altman plots for creatinine analyses. This shows a bias of 0.3589 ± 0.1057 for sealed blots, 1.126 ± 1.230 for unsealed blots, 1.818 ± 1.63 for sealed blots stored at 4 °C and 1.138 ± 1.341 for sealed blots stored at -20 °C and similarly the 95 % limits of agreement ranged from -1.713 to 2.431, -1.325 to 3.576, -1.378 to 5.013 and -1.491 to 3.766 respectively after 1 week of storage. Urine creatinine concentration is not significantly changed in the unsealed stored at room temperature and sealed stored at both room temperature, 4 °C and -20 °C for 7 months.

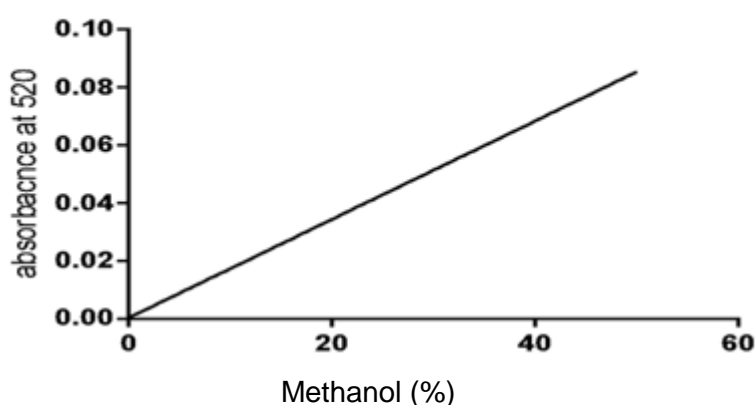


Figure 30: The impact of using methanol to extract the creatinine from a DPS

In order to justify omitting the deproteinising step in urine in the creatinine assay, creatinine was extracted from plasma in methanol but unusually high readings were observed and the lipid interference on the assay was checked. It was postulated that phosphoric tungstate delipidated the sample so methanol cannot be used to deproteinise (Figure 31).

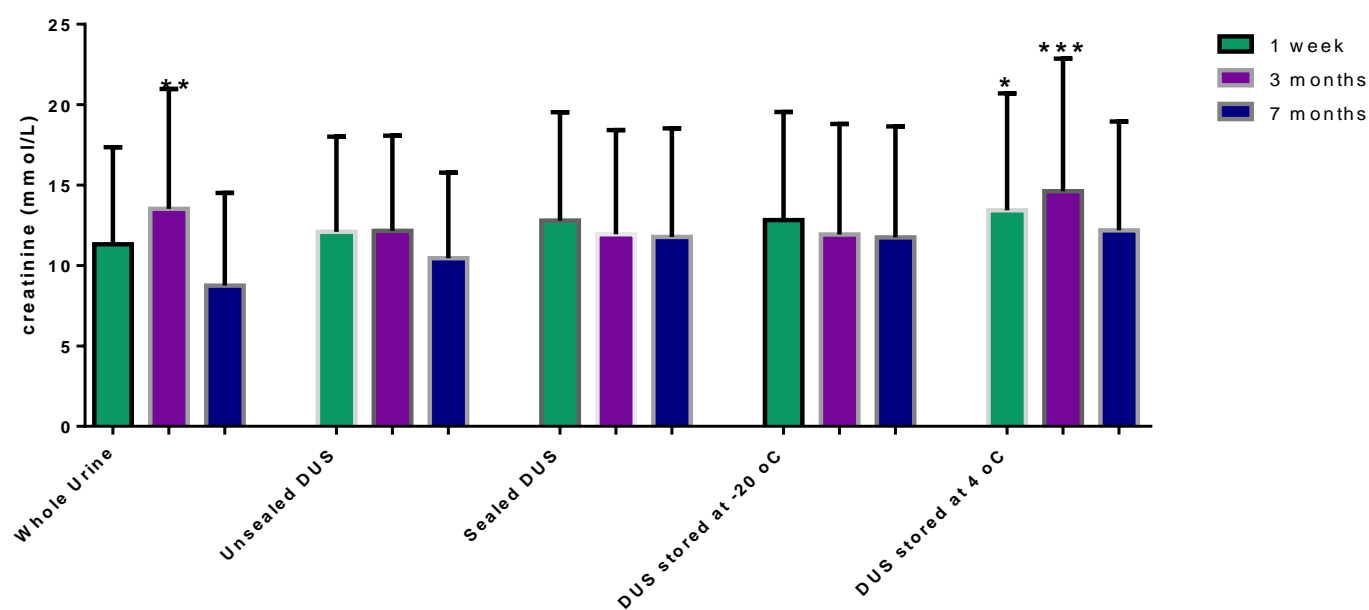


Figure 31: Comparison between creatinine concentration in whole urine and DUS samples stored at different temperatures and analysed 1 week, 3 months and 7 months after spotting. Green= samples analysed after 1 week of storage, purple= samples analysed after 3 months of storage and blue= samples analysed after 7 months of storage. * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$. Each of the spotted samples was compared to the original samples for statistical analysis

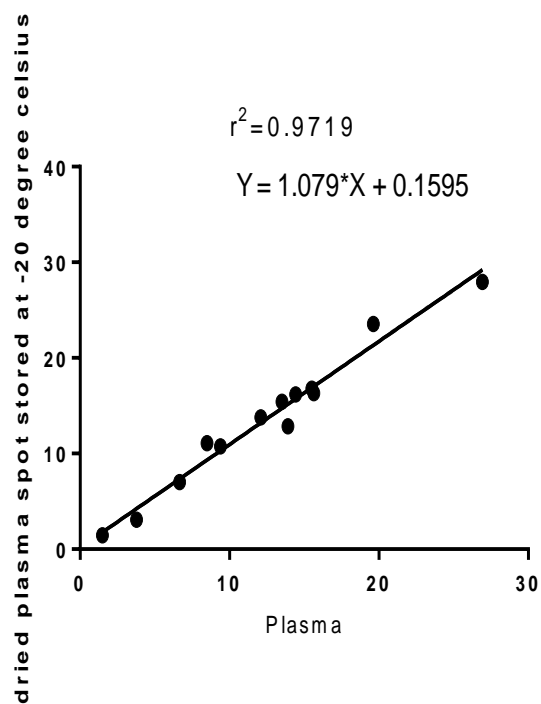
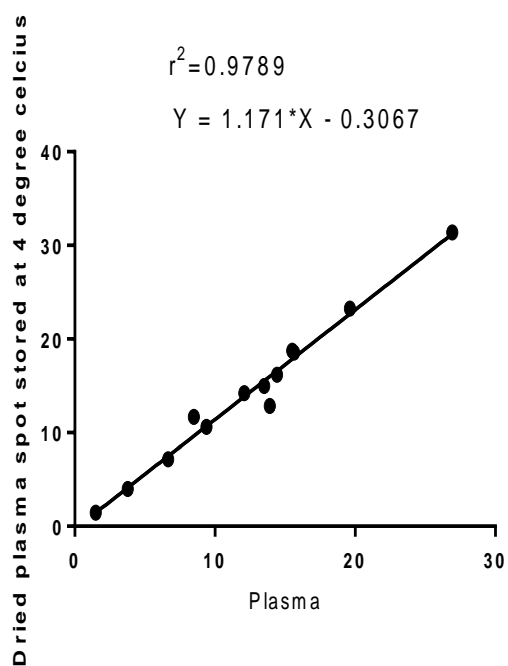
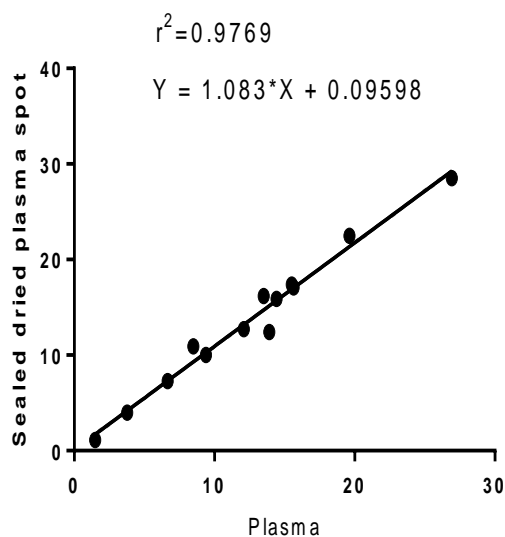
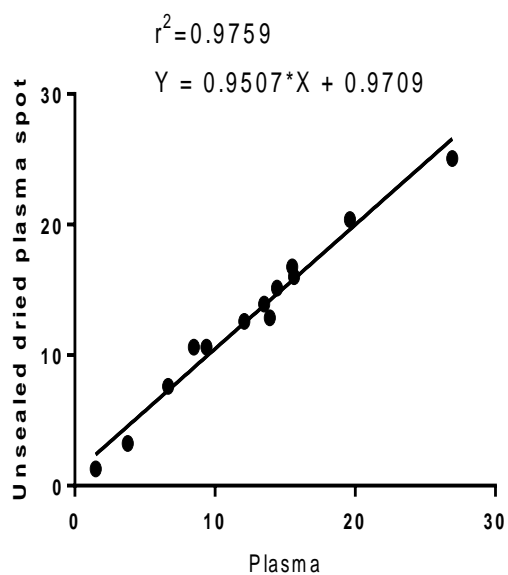


Figure 32: Correlation between creatinine concentration in urine and DUS samples stored at ambient temperature, 4 °C and -20 °C after a week of storage

4.8. Concentration of proteins in dried plasma spots and in whole plasma and PAGE of proteins

In clinical practice, dye binding assays such as bromocresol green or bromocresol purple are used for plasma protein assays but in research settings, the Lowry assay or related methods are typical (Wessel D and Flugge U.L, 1984). The protein concentrations of the plasma and dried plasma spots were determined using the Markwell modification of the Lowry protein assay (Markwell, M. A. K., et al., 1978).

The protein concentration of the unsealed DPS was significantly (paired non-parametric Anova, $p < 0.001$) lower than in whole plasma after 3 months and 7 months, respectively. The correlations between proteins concentrations in the DPS in unsealed, sealed at room temperature, sealed stored at 4 °C, sealed stored at -20 °C DPS and whole plasma were good after a week of storage but beyond that time, the correlations were not good. Figure 33 shows that the recoveries of extracted protein from DPS were good. However the amount of protein extracted from unsealed DPS was approximately 30 % lower than whole plasma after 1 week of storage and approximately 90 % lower of original samples after 3 months and 7 months of storage. But the sealed DPS stored at both room temperature, 4°C and -20 °C were highly correlated with the whole plasma, and the protein concentration was approximately 7 % more in whole plasma after 7 months of storage. Bland-Altman plots suggest that the difference in protein concentration for sealed DPS, irrespective of temperature and storage, and compared with whole plasma, was within 2 SD limits for most of the samples. It is unreliable to use DPS for determination of protein concentration using the protein modification on the unsealed DPS. The sealing the DPS helps in recovery of protein irrespective of the storage temperature.

Proteins were extracted from filter paper using different solvents (dH₂O, saline and NaOH) and electrophoresed on 20% acrylamide gradient gels. Figure 33 shows the comparison of proteins after being extracted from dried plasma spots and from whole plasma. The protein bands were very similar for whole plasma, plasma with saline and dH₂O.

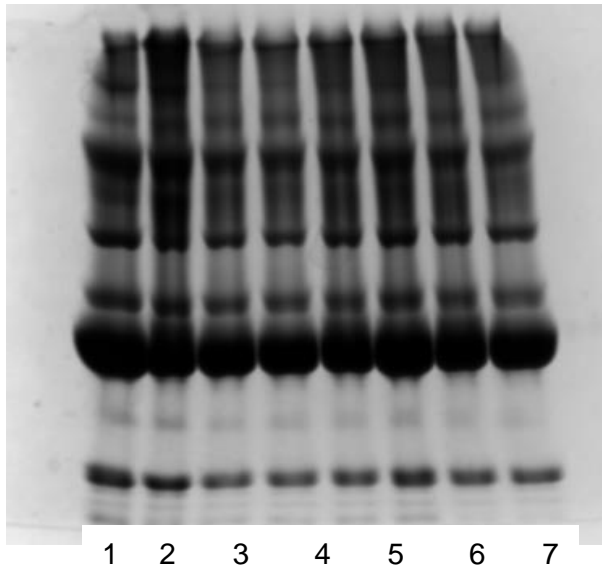


Figure 33: PAGE (5-20%) of proteins from DPS after a month of storage: Samples were load into the lanes as follows: lane 1 is whole plasma, lane 2 is whole plasma + 0.1% SDS, lane 3 is DPS + saline, lane 4 is DPS + saline + 0.1% SDS, lane 5 is DPS + dH₂O, lane 6 is DPS + dH₂O + 0.1% SDS, lane 7 is DPS + 0.1M NaOH, lane 8 is DPS + 0.1M NaOH + 0.1% SDS

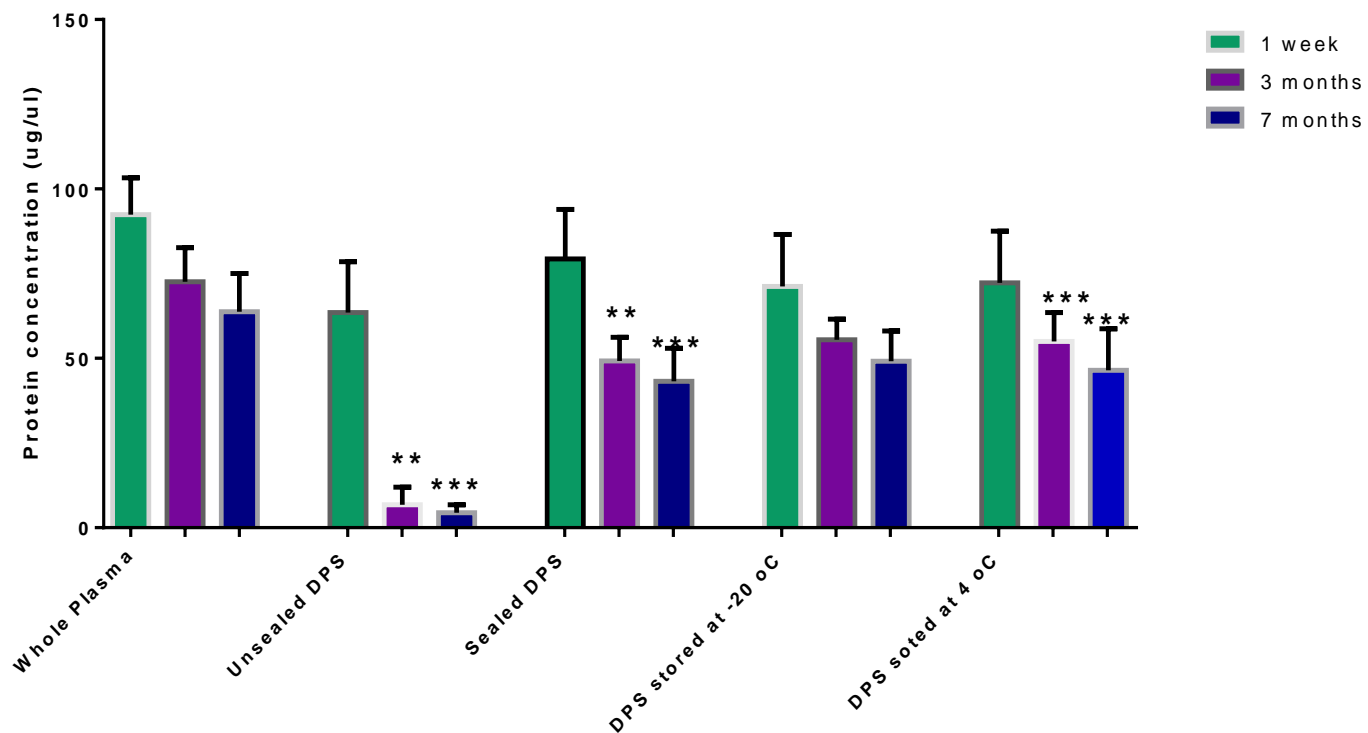


Figure 34: Comparison between protein concentration of whole plasma and DPS samples from random healthy participants stored at different temperatures for up to 7 months. Green= samples analysed after one week of storage, purple= samples analysed after 3 months of storage and blue= samples analysed after 7 months of storage. **p<0.001 and ***p<0.0001. Each of the spot samples was compared to the original samples for statistical analysis.

4.9. Concentration of salicylate in dried serum spots and in whole serum

To determine the utility of the DSS for therapeutic drug monitoring as well as toxicology in patients or those deceased, the whole serum from the 12 volunteers were spiked with sodium salicylate and spotted on the filter paper and stored for a week prior to analyses. The salicylate concentration in the whole serum and recovered DSS was then determined. The mean \pm standard deviation (SD) of salicylate concentrations obtained from DSS was 5.236 ± 2.361 $\mu\text{g}/\mu\text{L}$ and the mean \pm SD for the corresponding serum was 5.425 ± 2.501 $\mu\text{g}/\mu\text{L}$ (Figure 35). There was no significant difference between the DSS and whole serum. The relationship between DSS and whole serum was linear and highly correlated with an r^2 of 0.9887 after 1 month of storage. Figure 48 shows the Bland-Altman plots for salicylate analysis, which shows a bias for salicylate on filter paper spots of -0.1894 ± 0.2941 . The 95% Limits of Agreement ranged from -0.7658 to 0.387 . The intra- and inter-coefficients of variation of serum were 8.0 % and 7.6 % for DSS respectively. The DSS may reliably be used for determination of the salicylate concentration.

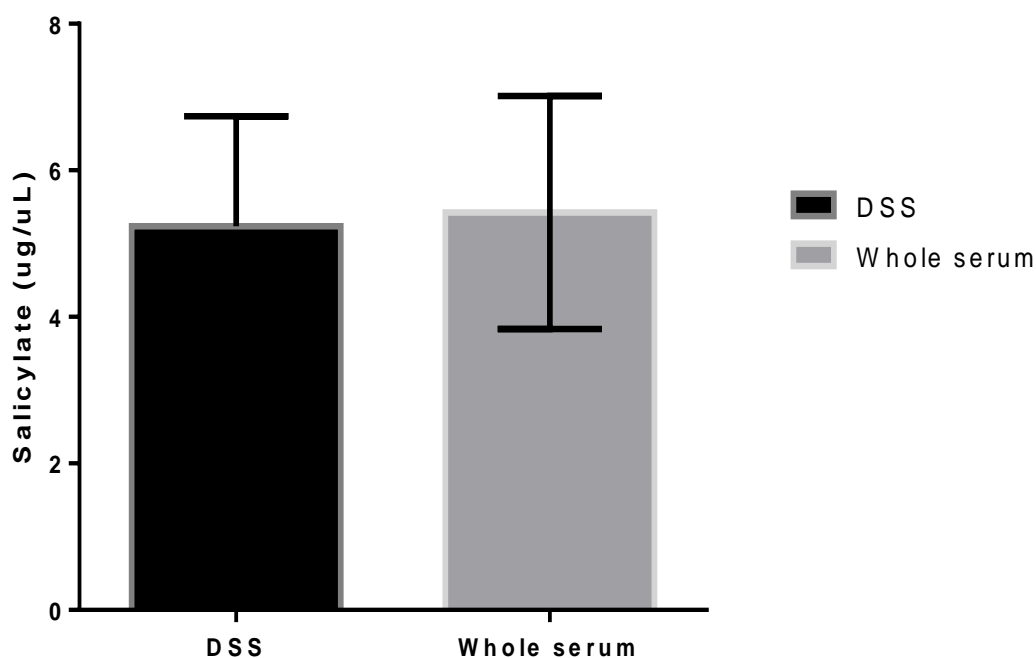


Figure 35: Comparison between salicylate concentration in whole serum and DSS samples after spiking the serum with sodium salicylate and storing for a week at room temperature. Non-parametric analysis was done.

4.10. A comparison of the concentration of cyanide in dried blood spots and in whole blood

To determine the utility of DBS (for example in forensic investigation of suspected cyanide poisoning) the whole blood was spiked with potassium cyanide spotted on the filter paper dried prior to extracting the equivalent volume of whole blood with saline into fresh microcentrifuge tubes. The mean \pm standard deviation (SD) of spiked cyanide for DBS was 20.86 ± 7.58 $\mu\text{mol/L}$ and for whole blood was 22.44 ± 7.30 $\mu\text{mol/L}$. There was no significant difference (paired t-test, $p>0.05$) between DBS and whole blood. The DBS was highly correlated with whole blood samples ($r^2=0.9026$) after a month of storage. The recovery of cyanide from DBS was 90%. The DBS is therefore reliable to determine the cyanide concentration, which therefore could be used in forensic science for investigations.

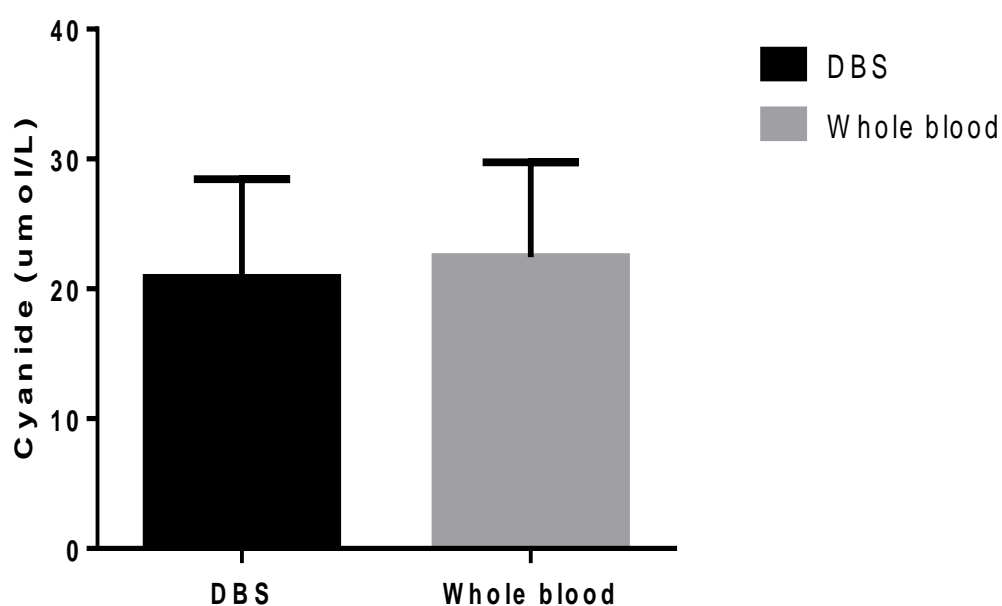


Figure 36: Comparison between cyanide concentration in whole blood and DBS samples. Non-parametric analysis was done.

Table 1: Results after drying serum and whole blood on the filter paper for analyses of salicylate (serum) and cyanide (whole blood) after 1 week of storage. Values are median (interquartile range)

	Original samples	DSS/DBS
Salicylate ($\mu\text{g}/\mu\text{L}$)	5.020 (3.36-7.91)	4.86(3.27-7.72)
Cyanide ($\mu\text{mol/L}$)	22.00(15.90-29.20)	24.00(12.95-27.20)

4.11. Thiobarbituric acid reactive substances (TBARS) with and without BHT on the filter paper

To determine if the DSS can be used for investigation of peroxidation of lipids, the serum from 10 volunteers was spotted on the filter paper, dried and analysed after 6 hours, 1 week and 1 month of storage at room temperature both sealed and unsealed and sealed at 4 °C and – 20 °C for CD and TBARS. The concentration of TBARS was determined 6 hours after spotting the serum onto filter paper using the Asakawa method (Asakawa T and Matsushita S, 1979). The TBARS concentrations on filter paper without BHT were significantly (paired non-parametric Anova, $p < 0.05$) higher than serum and spots with BHT. The results show that BHT protects lipids from peroxidation on the filter paper. The TBARS concentrations were significantly increased in the unsealed and sealed DSS without BHT both stored at room temperature and at -20 °C compared to whole serum and unsealed and sealed DSS with BHT, 6 hours and week of spotting but the difference disappeared after 1 month of storage.

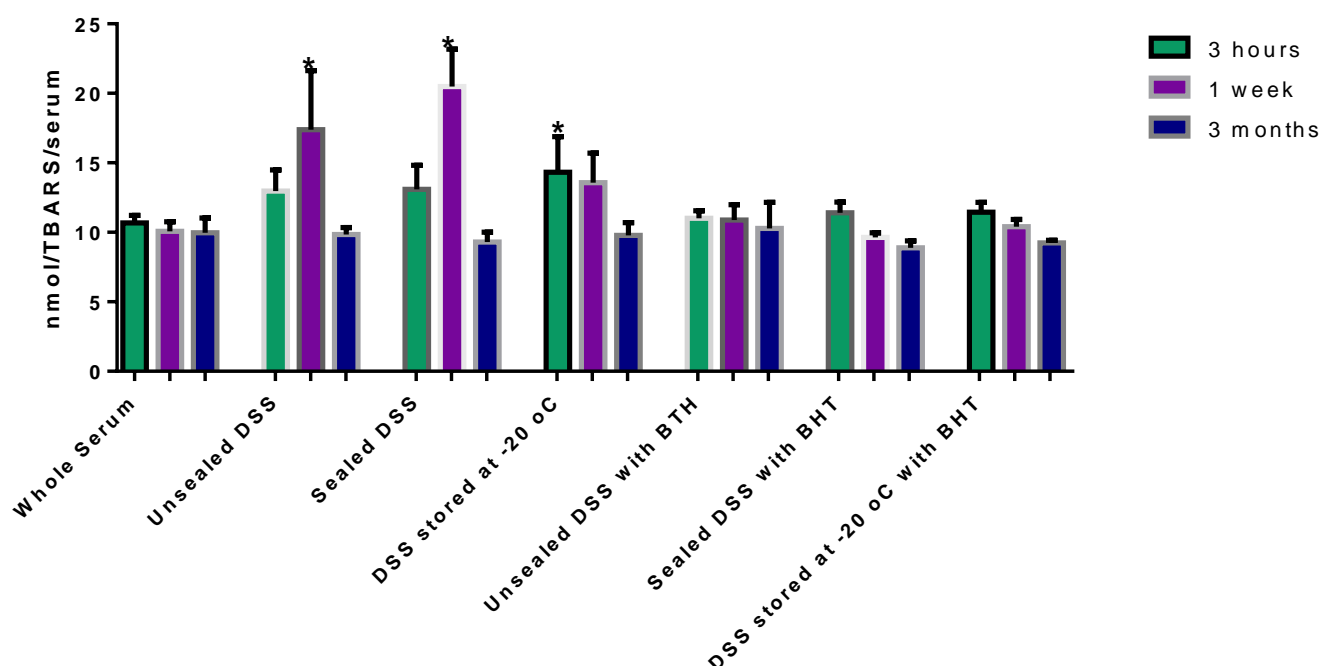


Figure 37: Comparison between TBARS concentration on week 0 after 6 hours of spotting the serum on the filter paper which was pre-soaked in 4 mmol/L of BHT and dried overnight, and filter paper without BHT. Green= samples analysed after 6 hours of storage, purple= samples analysed after 1 week of storage and blue= samples analysed after 1 month of storage. * $p < 0.05$. Each of the spot samples was compared to the original samples for statistical analysis. Nmol/TBARS/serum supposed to be nmol/L

4.12. Conjugated dienes (CD) with and without BHT on the filter paper

The concentration of CD in serum and DSS was determined after Folch extraction of the lipids, dried under nitrogen gas and re-dissolved in cyclohexane. The results are shown in Figure 38. The concentration of CD in the unsealed DSS without BHT increased by 30 % compared with that in serum after 7 days of storage, and by 22 % after 4 weeks of storage at room temperature. Sealed DSS without BHT increased by 14 % after 7 days storage at room temperature. The sealed DSS increased by 12 % after 7 days and by 18 % after 4 weeks at -20 °C. The DSS with BHT, CD increased by 20 % after 7 days with BHT on unsealed DSS, 11 % increased after storage at -20 °C (1 month of storage) and 3 % after 4 weeks of storage at room temperature. The filter paper which was pre-soaked in 4mmol/L in BHT/ ethanol showed the same CD concentration after 6 hours of drying the serum on the filter paper spot. The filter paper which was pre-soaked with 4 mmol/L BHT in ethanol and dried showed no

statistical difference of CD concentration after 6 hours compared to original serum. The CD were significantly increased (paired non-parametric Anova, $p<0.05$) in both sealed and unsealed DSS without BHT/ethanol stored at room temperature and $-20\text{ }^{\circ}\text{C}$, and unsealed DSS with BHT/ethanol stored at room temperature and after 1 week of spotting on the filter paper but the difference disappeared after a month of storage irrespective of whether filter paper was treated with BHT are not.

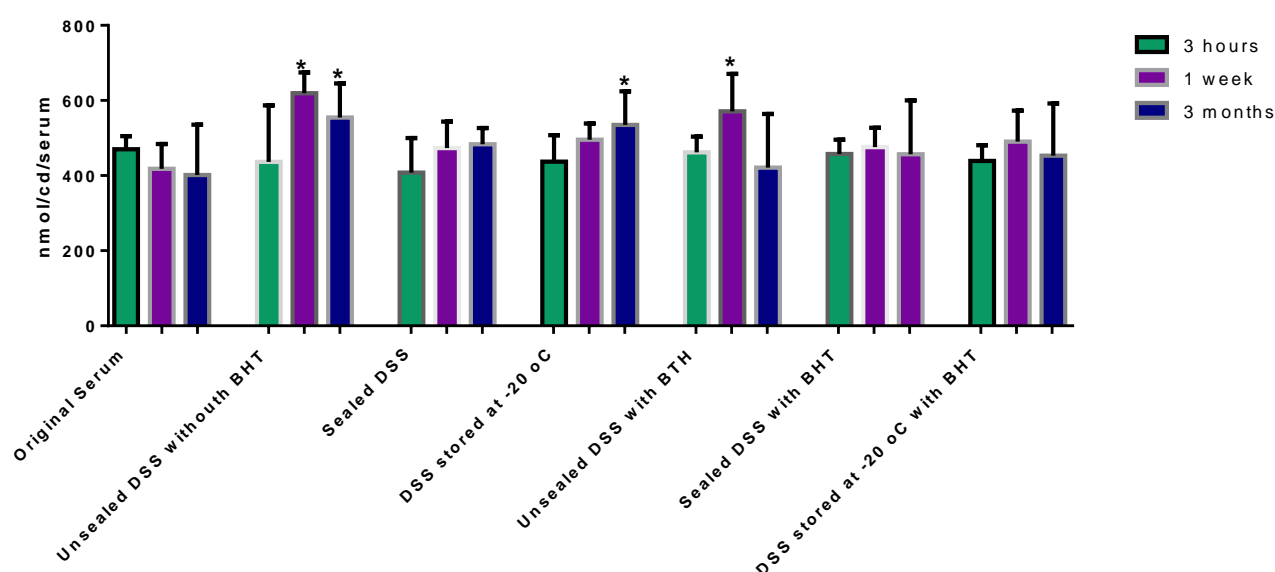


Figure 38: Comparison of CD concentration in whole serum and DSS stored at room temperature and $-20\text{ }^{\circ}\text{C}$ after 1 month. Green= samples analysed after 6 hours of storage, purple= samples analysed 1 week of storage and blue= samples analysed after 1 month of storage. * $p<0.05$. Each of the spot samples was compared to the original samples for statistical analysis. Nmol/CD/serum supposed to be nmol/L

4.13. DNA extraction and PCR

The concentration of extracted genomic DNA was measured as described in Materials and Methods (Section 3.9.14.) and the ratios of extracted DNA concentrations were calculated. As indicated in Figure 39, the recoveries of extracted genomic DNA from liquid and dried filter paper samples were compared. The amount of DNA extracted from whole blood was approximately 90 % higher than the yield of that from DBS, approximately 15 % higher than that from buffy coat and approximately 42% higher than that from dried buffy coat spots stored

at room temperature for a week. The A260/280 ratio is used to determine protein contamination of the nucleic acid (DNA and RNA) in the sample. It should be somewhere between approximately 1.8 and 2.0 and a ratio below 1.8 it indicates the contamination of nucleic acid (Desjardins P and Conklin D, 2010)) of extracted genomic DNA. The DNA extraction for whole blood, dried whole blood spot, buffy coat and dried buffy coat spot was assessed by the equation described in Section 3.9.14. The corrected A260 and A280 yielded ratios 1.55, 3.02, 1.63 and 1.47, respectively.

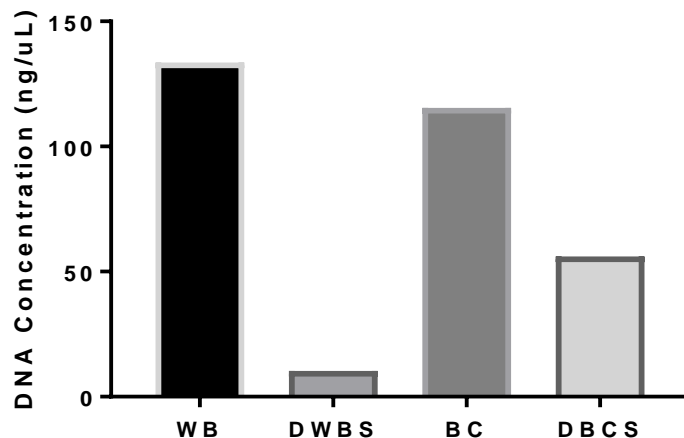


Figure 39: DNA recovery from liquid and dried samples. WB= whole blood, DWBS= dried whole blood spot, BC= buffy coat and DBCS= dried buffy coat spot

Genomic DNA from DBS and DBCS was extracted using the QIAamp® DNA mini kit purchased from Qiagen. The extract was subjected to PCR to find mutations in exon 2 of the STAP 1 gene using quantitative real-time PCR high-resolution melting technology (RT-qPCR-HRM). Samples were amplified on the LightScanner®32 (Idaho Technology Inc.), an HRM-enabled real-time PCR machine, and screened for sequence variations by curve analysis in a single run.

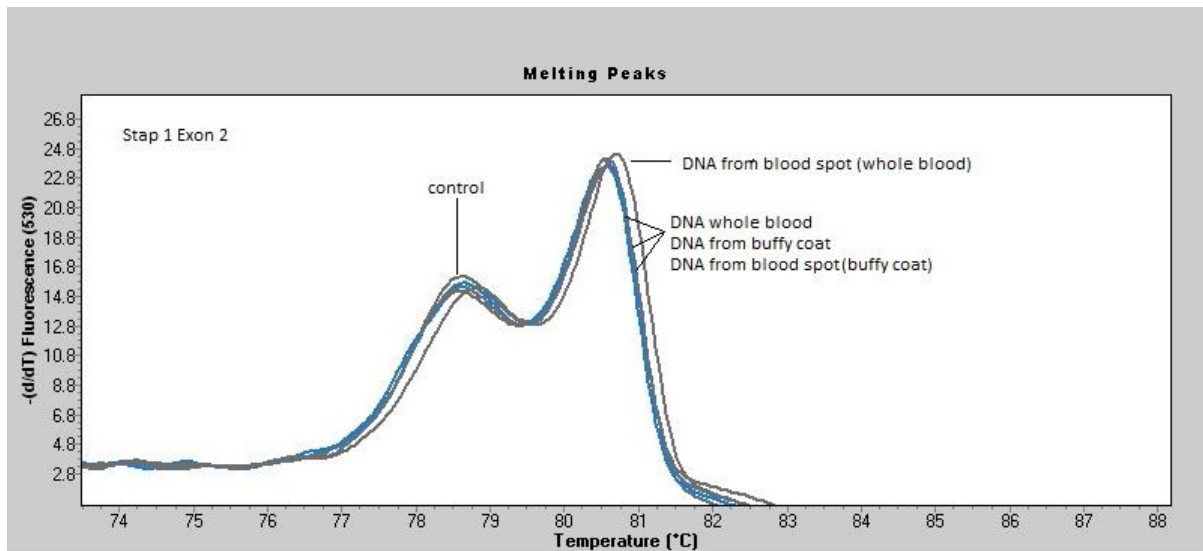


Figure 40: The melting peaks of the DNA extracted from whole blood, whole buffy coat, DBS and DBCS after PCR amplification of exon 2 of *Step 1*

The results showed that the genomic DNA extracted from dried buffy coat spots was of the same quality (to then be used in the PCR analysis) as those obtained from whole blood and buffy coat (Figure 40). However, the genomic DNA extracted from the DBS was only of fair quality as it behaved as a homozygous abnormal on the melting curve after amplification, even though the PCR was successful. Figure 41, shows the results of the DNA extracted on the filter paper having a higher melting peak compared to DNA extracted from whole blood.

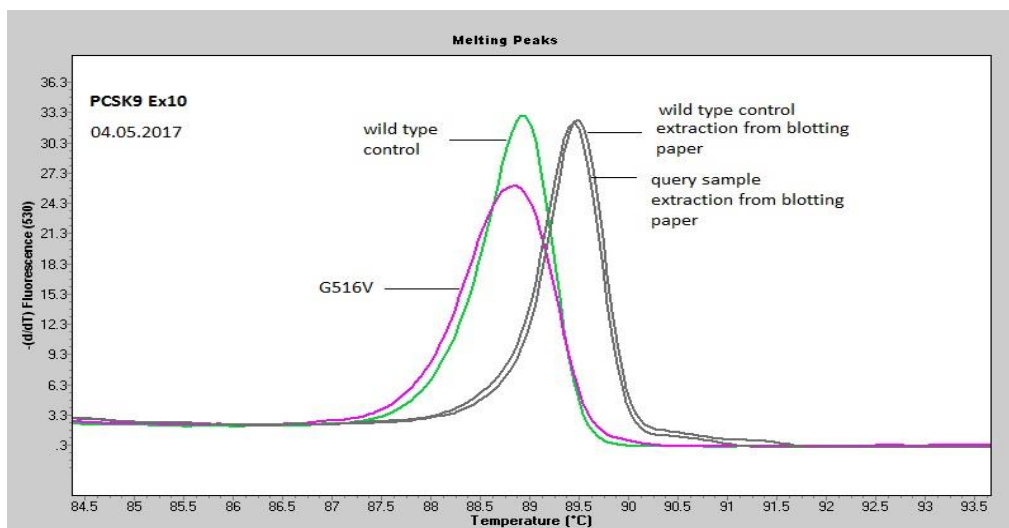


Figure 41: The melting peaks of the DNA extracted from whole buffy coat and DBCS after PCR collected from a patient with a Familial hypercholesterolemia (FH)

5. DISCUSSION

5.1. Introduction

The aim of this study was to develop a convenient, inexpensive, comprehensive and reproducible sample collection system on a single filter paper card that allows quantitative analysis of analytes in biological fluids for non-urgent common clinical laboratory and epidemiologic purposes as well as for specific investigation of metabolic, genetic and toxic conditions. An inexpensive commercially readily available filter paper was selected. Importantly, the dried sample is protected in a waterproof seal. The filter paper card is approximately the size of an A5 envelope to facilitate transport by regular mail, at a fraction of current costs of a courier and without the need for special treatment such as an ice box or dry ice. Additionally, there was exploration of the stability of lipid peroxidation markers (CD and TBARS) known to preserve poorly, for a month on the filter paper. Samples were spotted onto designated areas on the paper in prescribed volumes before drying, unsealed and vacuum-sealing. Storage was done at room temperature, 4 °C and -20 °C. Samples can be analysed multiple times. The sealed card can be banked so the patient need not return for more blood to be taken for further investigation. The dried sample is stable on the filter paper for 7 months or probably longer.

A comprehensive card of dried blood, buffy coat, serum, plasma and urine not only facilitates performing a range of tests but once-off collection of samples, avoids errors that may arise from repeat sampling of many patients and multiple tubes. The comprehensive range of samples is especially useful in working up metabolic disease where genetic confirmation may be desired after detection of metabolites in urine or serum. This is particularly important in South Africa where the intention is to improve healthcare to communities in rural areas that may not have received full attention to inherited metabolic disease in the past. The contribution to personalised medicine is another possibility.

5.2. Production and analysis of the dried spots

Production and analysis of the dried spots are conveniently discussed under the following headings.

- Design of the card
- Stability of analytes in spots
- Clinical laboratory analytes analysed
- Special analyses

5.2.1. Design of the card

Biologic fluids in common use for investigation are whole blood, buffy coat, plasma, serum and urine. Other sterile fluids could also be used for special purposes but samples with microbes may not be suitable unless special precautions are taken. All biologic samples must be considered as potentially infectious. Human immunodeficiency virus, hepatitis B virus and hepatitis C virus are common threats to laboratory workers. The personal protective equipment (PPE) (gloves, a laboratory coat or gown, and protective eyewear) should always be worn when handling and preparing the dried spots. The dried spot card and container should be marked as biohazardous.

The layout of the card should allocate adequate space and identification for the multiple samples, and appropriate information to identify the patient and the test(s) requested. The layout of the various fluids may vary depending on the clinic. For example in forensic cases, gastric fluid may be spotted for drug analysis. Many assays in modern laboratories require 2-10 μL of sample. Commercially available Guthrie cards generally have spots ranging from 10-15 mm in diameter. This card was thus designed with 15 mm diameter (176.7 mm^2) spots that, with a metal punch of 5 mm diameter (19.6 mm^2) would provide the equivalent of $3.7 \mu\text{L}$ of plasma. Up to 9 samples can be removed from such a spot if carefully punched. This may be more easily done if a triangular punch is used. The punching of 19.6 mm^2 is equivalent to $3.58 \mu\text{L}$ of whole blood and $1.9 \mu\text{L}$ of urine. The pencil marking of the paper is preferred as ink may affect some assays. The spotted filter paper should be sealed after drying. In this study the spots dried by 3 hours in agreement with general recommendations. But this may need to be confirmed under very cold or humid conditions. To obtain 15 mm spots, the following volumes were required: 60 μL of whole blood, 40 μL of plasma, 40 μL of serum, 60 μL of buffy coat and 20 μL of urine. A fixed volume pipette may simplify spotting the various samples without having to adjust the pipette. Multiples of 20 μL may be most convenient. The filter paper should be dried in a well-ventilated space for 3 hours and on a non-absorbing surface.

The blood is collected by venepuncture into EDTA tubes to obtain whole blood, plasma and buffy coat. One tube of 4.5 mL volume is adequate. For whole blood spotting, the blood should be mixed and spotted whereafter centrifugation is done to obtain plasma and after its removal, the buffy coat. Plain tubes (without additives) provide serum. A jar is used for the collection of urine.

Storage of dried spots in airtight, leak-proof containers (plastic or foil bags) was alleged to cause poor analyte stability and even degradation (Hannon et al., 2014). This project shows that vacuum-sealing the filter paper offers sufficient protection against immersion in water. Even though the sealing material was permeable to water, at ambient temperature, the movement was slow (0.08 mL/cm²/day). This rate will be slowed enormously when the sample is placed in a freezer. For improved storage several sealed cards may be placed in a sealed box containing desiccants. Analyte degradation was not observed on the dried spots sealed for 7 months in a freezer (-20 °C) and refrigerator (4 °C) without special precautions to limit exposure to air and moisture.

5.2.2. Stability of analytes in spots

This study examined a small number of analytes that are generally viewed as stable and a few analytes known to be vulnerable to oxidation (lipid peroxidation) but that can be protected by BHT.

The analytes were stable irrespective of whether the samples were stored at room temperature, 4 °C or -20 °C, with the exception of the total protein where unsealed spots showed a significant decrease in recovery after 3 months of storage. The explanation could be that crosslinking of proteins reduced their solubility. The stability of the analytes in this study was comparable with that described in a previous study (Lakshmy R et al., 2010). There was no degradation of plasma glucose, lactate, total protein and urinary creatinine at room temperature, both sealed and unsealed. A longer study needs to be carried out to establish how long the vacuum-sealed spots are stable without analyte degradation even though not much change is expected due to lack of air movement from the environment and the filter paper card. The stability of a more comprehensive set of analytes should be assessed before employing the card system in practice.

5.2.3. Clinical laboratory analytes

In this study, emphasis was given to non-urgent analytes commonly used in clinical practice and epidemiologic research for cardiovascular disease. Lactate was also evaluated because mitochondrial disease is one of the interests in the division of chemical pathology.

Several metabolic risk factors are well-recognised as predictors of CVD. Cardiovascular disease is responsible for much morbidity and premature deaths (Buitrago-Lopez A et al., 2011, Castro J.P et al., 2003). Impaired fasting glucose and type 2 diabetes mellitus are important CVD risk factors in clinical practice, especially because diabetes mellitus is on the rise. The WHO recommends a stepwise approach for measuring risk factors (WHO STEPwise). A lack of resources as well as capacity in developing countries, however, detracts from comprehensive screening. The dried spot cards offer a solution. In this study, good correlations, recoveries and stability were found when whole plasma and spots were analysed for glucose, lactate, triglyceride and protein. Recovery appeared incomplete for cholesterol and after 3 months for phospholipid.

Cholesterol is important for cardiovascular risk assessment. Future studies should investigate improving recovery or whether a correction factor is justified when the current method is sampled. The result for this study was consistent with Crimmins E et al., 2014 results that shown that cholesterol on DBS was less reliable compared to venous blood. Concomitant spotting of a standard on the card could also provide the most appropriate correction factor for cholesterol. The incomplete recovery was similar after storing at 4 °C and -20 °C (82 % and 81 %, respectively). The variation of the incomplete recovery was unacceptable for all dried spots irrespective of sealing and temperatures of storage. This makes it impossible for a sample correction for obtaining the true cholesterol in the original plasma.

The data shown in Figure 24 indicate significant increases in phospholipids between the original plasma and stored whole plasma. The reason is not clear. It is possible that thawing a frozen sample can float lipoproteins and thus increase their concentration at the top, unless mixing is complete. The assay is designed to release choline completely by enzymatic activity, so it is not expected that choline will be more accessible at later dates.

The traditional method for complete extraction of lipid is the Folch modification of Bligh and Dyer (Bligh E.G. and Dyer W.J, 1959). This procedure is feasible for dried spot analysis but requires more steps and time and could dilute the sample to the extent that the lipid is below the level of detection in the assay. The solubility of cholesterol in alcohol is reported as 1.29 % w/w at 20 °C and 28 % at 80 °C (Merck index, 1996). The extraction into methanol was expected to be adequate at 60 °C for all lipids of interest but it is possible that on cooling cholesterol precipitated. It is interesting to note that Metherel et al., 2013 used a similar methanolic extraction without loss of cholesterol. The suspension of cholesterol in methanol could be enhanced by a small amount of a miscible nonpolar solvent such as chloroform, or a

low concentration of non-ionic detergent that will not interfere with subsequent enzyme activity in the assay.

In this study, lipoproteins were not measured. Especially apo-B containing lipoproteins are not expected to remain intact and to be re-solubilised after desiccation.

Urine and DUS creatinine assays were comparable using the more elaborate method of Jaffe (Jaffe M, 1886). For plasma creatinine determination, it can be predicted that the enzymatic assay of creatinine would be much simpler than the method of Jaffe as no removal of protein and lipids is required. The enzymatic method was for sale only to laboratories with automated machines and could not be purchased for this study. For this reason plasma or serum creatinine evaluations were not performed. This study showed good recovery (107 ± 4.1 %) and correlation ($r^2=0.97$) of urine creatinine in spots compared with the original urine sample.

HbA1c has gained prominence in making the diagnosis of diabetes as well as monitoring control of this condition because it integrates glucose concentration over a long period. It was shown that a sample on the filter paper card can be stored for 44 days at room temperature before analysis by immunoturbidimetry, and up to 3 years at -70°C when analysed on HPLC (Egier D.A et al., 2011 and McDade TW et al., 2007). Since this assay is much more expensive than glucose assays, and is unlikely to receive support in practice in South Africa, HbA1c was not investigated.

The serum enzymes for determination of liver function were not assessed. It would, on first principles, be unlikely that enzyme activity will be restored well on extraction. Assays of bilirubin and albumin, however, should be feasible. The former is a colour reaction but for the latter, the colour reaction is more complex and vulnerable to chemical interference. Immunologic assays may be used but was not investigated. The plasma protein distribution was studied using PAGE. For research purposes, plasma proteins are often separated on PAGE and subsequently studied by western blots. This was not explored for this thesis. Agarose gel electrophoresis was not explored though this a conventional assay. Given that serum proteins are generally water-soluble it is likely that dried serum spots will be amenable to dissolution and analysis. Since lipoproteins are unlikely to redissolve this could influence the serum protein electrophoresis at the β , $\text{pre}\beta$ and α bands.

The intra- and inter-assay coefficients of variation for water-soluble analytes (creatinine, glucose, lactate and protein) on dried spots were greater than in whole plasma and urine.

These results are comparable with Lacher D.A et al., 2013 who showed the increased analytical variation of extraction from DBS and solubility of the analytes in the elution solvent.

5.2.4. Special analyses

Analyses of fatty acids and sterols, other cholesterol were not investigated. These are of great relevance in the evaluation of nutrition and metabolic errors. Of interest in lipidology are branched and very long chain fatty acids as well as dicarboxylic acids. Furthermore cholesterol precursors, bile salt intermediates and plant sterols are of interest. It is known that 7 dehydrocholesterol (Smith-Lemli-Opitz Syndrome) is unstable but adequate concentrations may persist to make this diagnosis (Kelley R.I and Hennekam R.C.M, 2000). The addition of BHT and storage under vacuum should protect against loss of 7 dehydrocholesterol. This needs confirmation with known positive controls.

Lipid peroxidation is of interest in an even narrower research field. Lipid peroxidation products are notoriously lost with time, even more so in films than in solutions due to their unstable state that causes them to change structure. Collection of the serum on the filter paper without BHT showed increased levels of CDs and TBARS 6 hours after spotting (see page 70, section 4.11. and 4.12.). Though prolonged storage is not recommended, this card system nevertheless was evaluated with more commonly used assays for lipid peroxidation. A good correlation and recovery was found between CDs and TBARS measured in dried serum spots on the filter paper which had been pre-treated with BHT and the original serum. Although BHT is usually applied to the sample for simplicity, in this study, the paper was treated with BHT. A previous study showed that spotting on filter paper pre-treated with BHT prevented the degradation of polyunsaturated fatty acids (PUFA) (Metherel A.H et al., 2013). This may increase the BHT concentration assimilated by diffusion of lipoproteins during the preparation of the spot. Conventionally, BHT is added to the sample to be preserved. This should be evaluated if the card is further developed as localised impregnation is technically demanding. The mechanism by which the BHT protects the lipids from oxidation on the spotted serum sample is through the phenol group donating a proton to free radicals. This protection could be further assisted by the vacuum seal which limits the movement of the air and thus the generation of free radicals to interact with the sample. The CDs were stable on the sealed pre-soaked filter paper for 1 month when stored at room temperature and at -20°C. Spotting the serum on pre-soaked filter paper with BHT without sealing, increased CDs significantly after 7 days of storage (Figure 37). The sequential increases in the concentrations of CDs and TBARS in the dried serum spots without BHT after a week of storage, followed by the

decreases after 4 weeks of storage are likely due to progression of PUFA peroxidation depleting double bonds and releasing volatile products.

The study carried out by Alberts et al (Alberts et al., 2005) at an impoverished South African rural area with inadequate and poorly accessible health care facilities found that 20 % of the population were at risk of developing CVD. They predicted a rise in prevalence in the next 10 years. This study showed that on the filter paper, glucose measurement was reliable compared with whole blood samples. Cholesterol in the current assay, is underestimated but could be still assist to improve the health care by detecting severe hypercholesterolaemia.

A wide range of genetic disorders is currently known. Early diagnosis and treatment may ameliorate the natural history of many disorders (Antonarakis S.E, 1989). Most of these disorders are not diagnosed at birth but develop over a few weeks when the neonate has already gone home. Genetic disorders affecting metabolism of amino acids, lipids, carbohydrates, and nucleic acids are individually rare but taken together are not rare. Except for patients presenting with acute and life threatening developments that need immediate diagnostic investigation, it would be ideal for investigations to proceed from primary health care by an inexpensive comprehensive system that would enable performing a range of biochemical and genetic tests. For urine a simpler card can be prepared by the mother who is instructed to press filter paper on the nappy for posting to a central laboratory. Visiting nurses or healthcare practitioners can use a heel prick for collection of blood for spotting and similarly they may place urine by means of a dropper on the card. An inexpensive home use vacuum sealer can be used at a clinic when the sample is dry. Centrifugation with portable devices including some that do not require electricity, is available and could be provided at clinics or even taken to homes.

The constraints on this project only permitted a limited assessment of genetic investigation as the chief focus was on chemical analytes. Tedious DNA extraction and purification is no longer required for most genetic tests as rapid preparation kits are commercially available. The 260:280 absorbance ratio of extracts from dried whole blood, whole blood, buffy coat and dried buffy coat was compared. The high ratio of 3.02 for the dried blood spot extract is puzzling. This value, unlike the low values in the other samples is not due to protein contamination. It is possible that turbidity interfered or that readings were at the lower limit of detection of the Nanodrop apparatus. As mentioned in section 3.9.14 the DNA extracted from whole blood, buffy coat and dried buffy coat spot with ratios of 1.55, 1.63, and 1.47 respectively, though not ideal, are workable. Accepting the calculated DNA content, the recovery of DNA from dried

whole blood spots was consistent with previous studies that showed a 10-15 % recovery as shown in Figure 39. The quantity of DNA extracted from dried buffy coat spots was higher than that from dried blood spots. This is expected because of the concentration of white cells increasing in the buffy coat. This amounted to 42 % compared to unspotted buffy coat. For this reason buffy coat is viewed as the desirable form of sampling DNA on the card. In this study the stability of the genomic DNA on the spots was not evaluated. A previous study showed no difference in the quality of DNA after 2 years of storage but after 3 years extraction yielded less genomic DNA (Harota T et al., 1989). However, unlike in this study sealing of the samples was not done and the samples were not stored at -20°C .

Irrespective of the amount of DNA and its quality, the ultimate criterion is that a genetic test is reliable. Typically, genotyping requires amplification of the segment of DNA of interest by using appropriate primers. This amplicon could be analysed by various means. Originally restriction enzyme analysis was convenient for many mutations but high resolution melting became a more convenient method to detect mutations. This method, using Sybergreen/LC green/ Eva green dyes relies on fluorescence of the dye in double stranded but not single stranded DNA. This process may be very sensitive to matrix effects. Caution should be exercised when using HRM to detect mutations. To overcome confusion from matrix effects, samples with known mutations should be analysed from filter paper. The melting peak of the genomic DNA extracted from the dried whole blood spots was compared with that of extracts from whole blood. Similarly, DNA from the buffy coat and dried buffy coat spot was compared.

PCR followed by HRM was successful in producing amplicons in a study of FH due to mutations in *STAP-1* (Figure 41). It can be demonstrated that the curves for whole blood, buffy coat and dried buffy coat spots are identical and very close to the reference DNA. As shown in Figure 40, the whole blood spot extraction of DNA yielded a slightly different HRM curve than the other preparations. The peak was shifted by about 0.25°C . This appears significant given that the other 3 samples prepared identically, overlapped perfectly.

Wild type and a known mutation in PCSK9 was investigated in two family members (Figure 41). The matrix effect appeared stronger in raising the peak melting temperature but there was no difference in the HRM pattern between the wild type control and that of the family member. Blood was not available from the index case for the ideal comparison with dried buffy coat

spot but the wild type and affected family member displayed such identical patterns that the conclusion is viewed as correct.

Testing for cyanide and salicylate was to evaluate the card for toxicology and in the latter case also for therapeutic monitoring. The results for dried whole blood with cyanide and serum spots with salicylate showed that the card system could indeed be useful in forensic investigation of poisoning or drug testing. The compounds in the original samples are likely to be less stable during storage. Since some degradation may occur in fluids rather than on the filter paper, keeping sealed cards of dried multiple body fluids makes for better preservation and less contamination.

5.2.5. Limitations of study

1. The range of analytes evaluated was limited and should be expanded to accommodate most of the tests requested by clinics. The samples from the volunteers were in the normal reference range. Abnormal high and low values were not studied. Only small sample numbers were analysed and thus the overall reproducibility and stability cannot be assessed.
2. The card was not tested in the field. Ideally, selected, if not all tests should be compared between a conventional (wet) assay and an assay from a card that was prepared and sent by post. Additionally, comparisons should be done with a conventional Guthrie card to indicate that both types of filter paper are suitable. The original recommended filter paper (Whatman 903) for spot analysis could not be sourced in unmarked sheets and presented a significant cost.
3. The layout was not determined for the best locations and numbers of spots. Incomplete recovery of cholesterol was not investigated nor was the detection of lipoprotein though it is not expected that lipoproteins will survive desiccation.
4. Chromatography was not pursued on extracted samples: FAME by GC-FID, sterols by GC-FID, drugs by LC-MS.

5.3. Future recommendations

1. Consultation should be undertaken with stakeholders to establish their needs and ensure that the commonest requests are incorporated to provide as complete a service

- as possible. Consideration should be given to broadening the repertoire to include infectious disease.
2. The measurement of minerals and electrolytes could be assessed on the filter paper, bearing in mind that the filter paper needs to be very clean and handling of the card should not expose it to contamination.
 3. Optimising the number of spots on the filter paper needs attention. The layout and allocation of spots for particular fluids should be adapted according to how many assays are normally done but with reasonable amounts of other samples available should testing be extended. This will avoid additional demands on the referring clinic and patient.
 4. Exploration of other investigations (liquid or dissolved solids) to be spotted on a blank area. This could include testing the quality of medication or identifying unknown medication (s) or supplements. The extraction could be used for spectrophotometry, fluorometry and chromatography.
 5. Expansion of genetic testing not only for inherited disorders, but also testing for infection (malaria and HIV).
 6. Once the details of the card are worked out, and mass application is envisaged, consideration should be given to designing and manufacturing a portable device that expedites the spotting drying, recording and sealing of the card. A box that provides lighting of the card with areas to be spotted correctly and a mesh for receiving a card that will be ventilated by a fan and heater can standardize and speed up drying. The box could also accommodate a UV light to indicate the border of urine spots for pencilling in. This device should be accompanied by a small centrifuge so that an EDTA or plain tube (without additives) can be processed to prepare samples for spotting. A robust pipettor that can be simply adjusted in intervals of 20 μ L will ensure ease and speed of spotting of the various samples. The sealing device could be accommodated in the main box or a robust free-standing device can accompany it. The cards can be pre-prepared with bar codes. Ideally, they should also have a small strip of paper giving the most salient details about the patient and a small space for annotating the use of material from the card. This could be developed on a commercial basis for Africa where healthcare needs to be improved but where it is unlikely that laboratories will be in easy reach of most clinics in the foreseeable future.
 7. Screening and diagnosis in future could be simplified by Direct Analysis in Real Time (DART) of a plasma or urine sample or a simple extract into methanol from a spot.

5.4. Conclusion

A comprehensive practicable card system was investigated for a range of tests that are required in various fields of investigation. These include epidemiologic studies, clinical laboratory tests, metabolic disorders and genetic tests, therapeutic monitoring as well as toxicological and infectious disease tests. This system can be explored further for reliability, reproducibility and range of tests. If found useful, then a special device for preparing the cards could significantly improve healthcare in isolated areas. Overall, a comprehensive, inexpensive and robust card sampling system using regular mail or other means of transport can vastly improve healthcare and research in Africa.

6. APPENDICES

Appendix I:

Participant information Sheet and Consent Form:

Statement concerning participation in a Clinical Research Project

Title of Project:

DRIED SPOT CARDS TO ANALYSE BIOLOGIC FLUIDS FOR DIAGNOSTIC INVESTIGATION OF PATIENTS

Participant name:.....

Thank you very much for taking your time to read this document. If there is anything you do not understand in this Document, please feel free to ask one of the researchers to explain it to you. We would like to invite you to take part in a research study called “Dried spot cards to analyse biologic fluids for diagnostic investigation of patients’. If you agree to take part in this study we will ask you to sign this consent document. You will be given a signed copy of this document to take home with you. This study will be conducted according to the ethical guidelines and principles of the Declaration of Helsinki (2013) and South African Good Clinical Practice Guidelines. This study has also been approved by Human Research Ethics Committee of the University of Cape Town.

Explanation of Research study

Dried blood spot is a method where biologic fluids (whole blood, plasma, serum, buffy coat and urine) are spotted on to filter paper and dried. The spotting of biologic fluid on filter paper required little volume of blood. Samples will be sent to the laboratory in dried form where analyte will be eluted from the filter paper by solvent and analysed.

Currently, collection of samples for laboratory analysis of whole blood, plasma, serum and urine requires sampling in fragile tubes, usually with additives: for example ethylenediaminetetraacetic acid (EDTA) or citrate. The analytes or matrix may be unstable so testing needs to be carried out quickly. This necessitates prompt or special transport on ice or dry ice by vehicle or courier services, in order to maintain sample integrity. Usually, in diagnostic laboratories, test tubes impose storage and time constraints which would be overcome by receiving samples on the proposed card system for this project which will have a variety of fluids in the appropriate matrix for analysis.

It is therefore desirable to establish a technique that will involve various samples from the same person being collected on filter paper in which the dried analytes will be stable and available for diagnosis. The samples will include for example blood and urine. These will be collected on filter paper and subsequently dried.

Your participation in this study is entirely voluntary. If you decide not to take part this will in no way impact on the care you receive from clinic or hospital.

What will happen if I decide to take part in the study?

You will take about 5ml of blood (one teaspoon / one small blood tube) from you.

Is there any risk taking part in this study?

The risk of taking part in this study is minimal as it will only involve taking one (or in some cases two) blood sample(s). Taking a blood sample may cause some pain as the needle enters the skin. There may also be some bruising or bleeding at the site of the needle prick and very rarely infection can occur. Some people may feel faint when having their blood taken.

Is there any benefit to me in taking part in this study?

Taking part in this study will not benefit you directly. The information obtained from this study may help to improve the diagnosis and treatment of patients with abnormal blood fats in the future. You will not be paid to take part in this study.

Confidentiality and sample storage

All information from this study will remain strictly confidential, all sample tubes will be labelled using alphabets. If the Human Research Ethics committee of the University of Cape Town decides to inspect this study they will be given access to your consent form as well. Your blood sample will be stored for the duration of the study in case needs to repeat the laboratory test we are studying but will be destroyed once the research study is complete.

If you have questions about this study please feel free to ask one of the researchers.

Mr A.M. Rapulana (Master's candidate)

Dr D. Blackhurst (Supervisor) or Prof A.D. Marais (co-supervisor)

Clinical laboratory Sciences

Division of Chemical Pathology-Lipodology

6th floor, Falmouth Building

UCT Medical School- Faculty of Health sciences

Anzio Road

7925

Tel: 021 406 6108/6185

If you feel that your questions have not been answered adequately or you have any other concerns you can contact the Human Research Ethics Committee of the University of Cape Town at:

Professor M. Blockman (Chairperson)

Human Research Ethics Committee
E52 Room 24, Old Main Building
Groote Schuur Hospital
Observatory
7925
Tel: 021 406 6492

I have heard the aims and objectives of the proposed study and was provided the opportunity to ask questions and given adequate time to consider. The aim and objectives of the study are sufficiently clear to me. I have not been pressurised to participate in any way.

Yes ☐ No ☐

I am aware that this material may be used in scientific publications which will be electronically available throughout the world. I consent to this, knowing that my identity is not revealed.

Yes ☐ No ☐

I understand that participation in this Project is completely voluntary and that I may withdraw from it at any time and without supplying reasons. This will have no influence on the regular treatment that holds for my condition neither will it influence the care that I receive from my regular doctor.

Yes ☐ No ☐

I know who to contact if I have any questions about the study in general

Yes ☐ No ☐

I understand my responsibilities as a study from the study

Yes ☐ No ☐

I hereby give consent to participate in this Project.

Yes ☐ No ☐

.....
Name of participant/volunteer

.....
Signature of patient or guardian.

.....
Place.

.....
Date.

.....
Witness

Statement by the Researcher

I provided verbal and/or written information regarding this Project.

I believe that the participant understands the study and has given informed consent to participate.

I agree to answer any future questions concerning the Project as best as I am able.

I will adhere to the approved protocol.

.....
Name of Researcher	Signature	Date	Place

Appendix II

1. Cholesterol assay (Cholesterol standard 200 mg/dL)

a. Cholesterol standard curve

25 μ L methanol + 25 μ L dH₂O = 0 μ g

1 μ L cholesterol standard + 25 μ L methanol + 24 μ L dH₂O = 2 μ g

2 μ L cholesterol standard + 25 μ L methanol + 23 μ L dH₂O = 4 μ g

3 μ L cholesterol standard + 25 μ L methanol + 22 μ L dH₂O = 6 μ g

5 μ L cholesterol standard + 25 μ L methanol + 20 μ L dH₂O = 10 μ g

7 μ L cholesterol standard + 25 μ L methanol + 18 μ L dH₂O = 14 μ g

10 μ L cholesterol standard + 25 μ L methanol + 15 μ L dH₂O = 20 μ g

13 μ L cholesterol standard + 25 μ L methanol + 12 μ L dH₂O = 26 μ g

16 μ L cholesterol standard + 25 μ L methanol + 11 μ L dH₂O = 32 μ g

2. Triglyceride assay (Standard solution (glycerol corresponding to 300 mg/dL triolein))

a. Triglyceride standard curve

25 μ L methanol + 25 μ L saline = 0 μ g

1 μ L standard solution + 25 μ L methanol + 22 μ L saline = 3 μ g

2 μ L standard solution + 25 μ L methanol + 23 μ L saline = 6 μ g

5 μ L standard solution + 25 μ L methanol + 20 μ L saline = 15 μ g

7 μ L standard solution + 25 μ L methanol + 18 μ L saline = 21 μ g

10 μ L standard solution + 25 μ L methanol + 20 μ L saline = 30 μ g

15 μ L standard solution + 25 μ L methanol + 10 μ L saline = 45 μ g

20 μ L standard solution + 25 μ L methanol + 5 μ L saline = 60 μ g

b. Saline (0.9 % NaCl)

0.9 g of NaCl + 100 mL of dH₂O

3. Phospholipid assay (Phospholipid standard (Choline Chloride 54 mg/dL corresponding to 300 mg/dL phospholipid))

a. Phospholipid standard curve

25 μ L methanol + 25 μ L dH₂O = 0 μ g

1 μ L phospholipid standard + 25 μ L methanol + 24 μ L dH₂O = 0.541 μ g

2 μ L phospholipid standard + 25 μ L methanol + 23 μ L dH₂O = 1.082 μ g

3 μ L phospholipid standard + 25 μ L methanol + 22 μ L dH₂O = 1.623 μ g

5 μ L phospholipid standard + 25 μ L methanol + 20 μ L dH₂O = 2.705 μ g

8 μ L phospholipid standard + 25 μ L methanol + 17 μ L dH₂O = 4.328 μ g

10 μ L phospholipid standard + 25 μ L methanol + 15 μ L dH₂O = 5.41 μ g

15 μ L phospholipid standard + 25 μ L methanol + 10 μ L dH₂O = 8.115 μ g

20 μ L phospholipid standard + 25 μ L methanol + 5 μ L dH₂O = 10.82 μ g

25 μ L phospholipid standard + 25 μ L methanol = 13.525 μ g

4. Glucose assay (glucose standard I 200 mg/dL and glucose standard II 500 mg/dL)

a. Glucose standard curve

0 μ L of glucose standard I + 20 μ L dH₂O = 0 μ g

0.5 μ L of glucose standard I + 19.5 μ L dH₂O = 1 μ g

1 μ L of glucose standard I + 19 μ L dH₂O = 2 μ g

2 μ L of glucose standard I + 18 μ L dH₂O = 4 μ g

1 μ L of glucose standard II + 19 μ L dH₂O = 5 μ g

3 μ L of glucose standard I + 17 μ L dH₂O = 6 μ g

4 μ L of glucose standard I + 16 μ L dH₂O = 8 μ g

2 μ L of glucose standard II + 18 μ L dH₂O = 10 μ g

The rest of the method is described in 3.9.4

5. Creatinine assay

a. Creatinine standard curve

0 μ L creatinine standard + 50 μ L dH₂O = 0 μ g

0.5 μ L creatinine standard + 49.5 μ L dH₂O = 0.05 μ g

1 μ L creatinine standard + 49 μ L dH₂O = 0.1 μ g

5 μ L creatinine standard + 45 μ L dH₂O = 0.5 μ g

10 μ L creatinine standard + 40 μ L dH₂O = 1 μ g

20 μL creatinine standard + 30 μL dH_2O = 2 μg
 30 μL creatinine standard + 20 μL dH_2O = 3 μg
 40 μL creatinine standard + 10 μL dH_2O = 4 μg
 50 μL creatinine standard + 0 μL dH_2O = 5 μg

6. Markwell modification of Lowry method

a. Bovine Serum Albumin standard (at 1 $\mu\text{g}/\mu\text{L}$ BSA)

100 μL of BSA standard = 100 μg
 0 μL of BSA standard + 100 μL of dH_2O = 0 μg
 10 μL of BSA standard + 90 μL of dH_2O = 10 μg
 20 μL of BSA standard + 80 μL of dH_2O = 20 μg
 30 μL of BSA standard + 70 μL of dH_2O = 30 μg
 40 μL of BSA standard + 60 μL of dH_2O = 40 μg
 50 μL of BSA standard + 50 μL of dH_2O = 50 μg
 60 μL of BSA standard + 40 μL of dH_2O = 60 μg
 70 μL of BSA standard + 30 μL of dH_2O = 70 μg
 90 μL of BSA standard + 10 μL of dH_2O = 90 μg

b. Solution A (2 g/mL anhydrous Na_2CO_3 ; 0.4% NaOH ; 0.16 g/mL $\text{Na}_2\text{Tartrate}$; 1 g/mL SDS)

c. Solution B (4 g/mL $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)

d. Solution C

1:1 Folin Ciocalteu to dH_2O

e. 0.1 M NaOH

0.4 g of NaOH was added to a beaker and dH_2O was added up to 10 mL

The rest of the method is described in Section 3.9.7.

7. POLYACRYLAMIDE GEL ELECTROPHORESIS

A 5 to 20 % gradient polyacrylamide gel was prepared. Using a gradient maker 5 % polyacrylamide was made up of acrylamide solution to which was added, 0.66 mL of 30 % acrylamide solution was pipetted in to 50 mL tube and 2 mL of 0.1 % SDS solution. 1.34 mL of buffer B was also added into the same centrifuge tube. 20 % polyacrylamide was made up of 2.66 mL of 30 % solution to which was added and buffer A. Into each tube 5 and 20 % polyacrylamide was added 40 μL of ammonium persulphate (10 g%) and 10 μL of

Tetramethylethylenediamine (TEMED) and gel was poured between 2 glass plates of a Bio-Rad chamber and allowed to set.

3 % stacking gel was also prepared by adding 0.5 mL of 30 % acrylamide and 4.5ml of stack buffer and the tube was mixed by gently swirling the tube, and 100ul of ammonium persulphate and 25 μ L of TEMED were added and the solution was again mixed by swirling. The stacking gel was pipetted on top of an already set 5-20 % acrylamide gel and a comb to generate wells of the required volume was inserted into the stacking gel. The stacking gel was allowed to set, and the comb was gently removed and the electrode buffer poured onto the gel which had been transferred to electrophoresis apparatus.

a. Acrylamide monomer solution

150 g of acrylamide, 4 g Bisacrylamide and 0.5 g of SDS was weighed and placed in the measuring cylinder and 500 mL of dH₂O was added

b. Gel Buffer A

68.1 g of Tris base, 150 mL and 0.5 g SDS was weighed and placed into a measuring cylinder and 500 mL of dH₂O was added. Dissolved the mixture and adjust pH to 8.8

c. Gel Buffer B

68.1g of Tris base and 0.5g of SDS was weighed and placed and placed into a Measuring cylinder and dH₂O was added up to 500mL. Dissolved and adjust pH to 8.8
Separation Gel Overlay

0.375 M Tris base, 0.1 % SDS at pH to 8.8. This is to cover the separation gel while it is setting in preference to water. For 100mL. Tris 4.5g and 0.1g SDS

d. Stack Gel Buffer

8.4 g of Tris base and 0.5 g of SDS was weighed and placed into a beaker

e. Ammonium Persulphate

0.1 g of ammonium persulphate was weighed and add 1 mL of dH₂O

f. TEMED

Dilute tetramethylethylenediamine 10x with dH₂O: 20 μ L of TEMED was added to 180 μ L dH₂O

g. Electrode buffer stock (x5)

Weigh 37.94 g of Tris base; 180.1 g of Glycine and 12.5 g SDS and 2.5 L of dH₂O. Dissolve and adjust pH to 8.3

h. Sample Treatment Buffer (x4)

1.518 g of Tris base, 2 g of SDS was weighed and 20 mL of glycerol and dH₂O was added up to 50 mL

i. Coomassie Stain

Coomassie stain stock (measure 2 g of coomassie blue R-250 into a 50 mL beaker and add about 20 mL of dH₂O and stir in the dye. 100 mL was transferred volumetric flask and add deionized water up to the line (meniscus))

j. Destain I

Measure 500 mL of methanol, 100mL of acetic acid and 400 mL of dH₂O and mix.

k. Destain II

Measure 70 mL of methanol, 50 mL of acetic acid and 880 mL of dH₂O and mix.

Appendix III

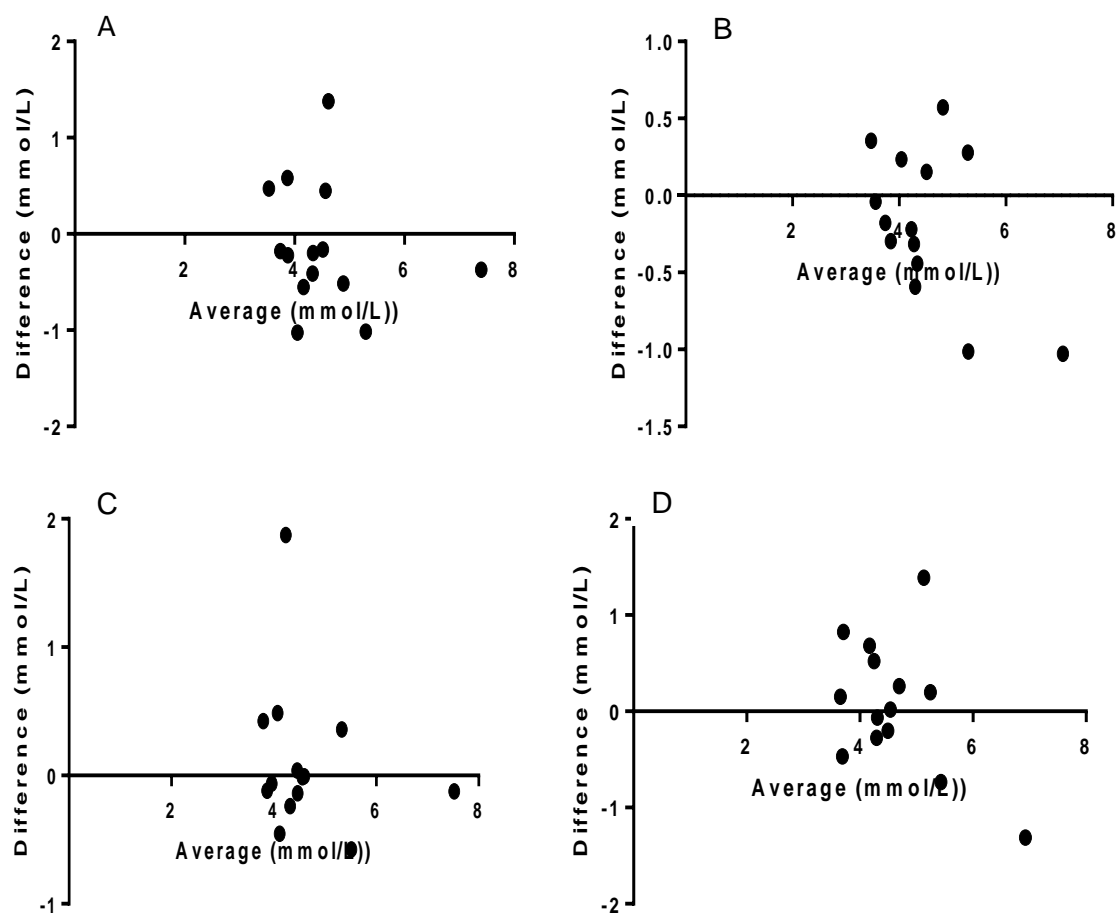


Figure 42: Bland-Altman plot of difference in cholesterol values obtained from fresh plasma and dried plasma spots (spotted immediately after collection) stored at ambient temperature after 7 days of blood collection, A = Sealed spot, B = Unsealed spot, C = Sealed stored at 4 °C and D = sealed stored at -20 °C

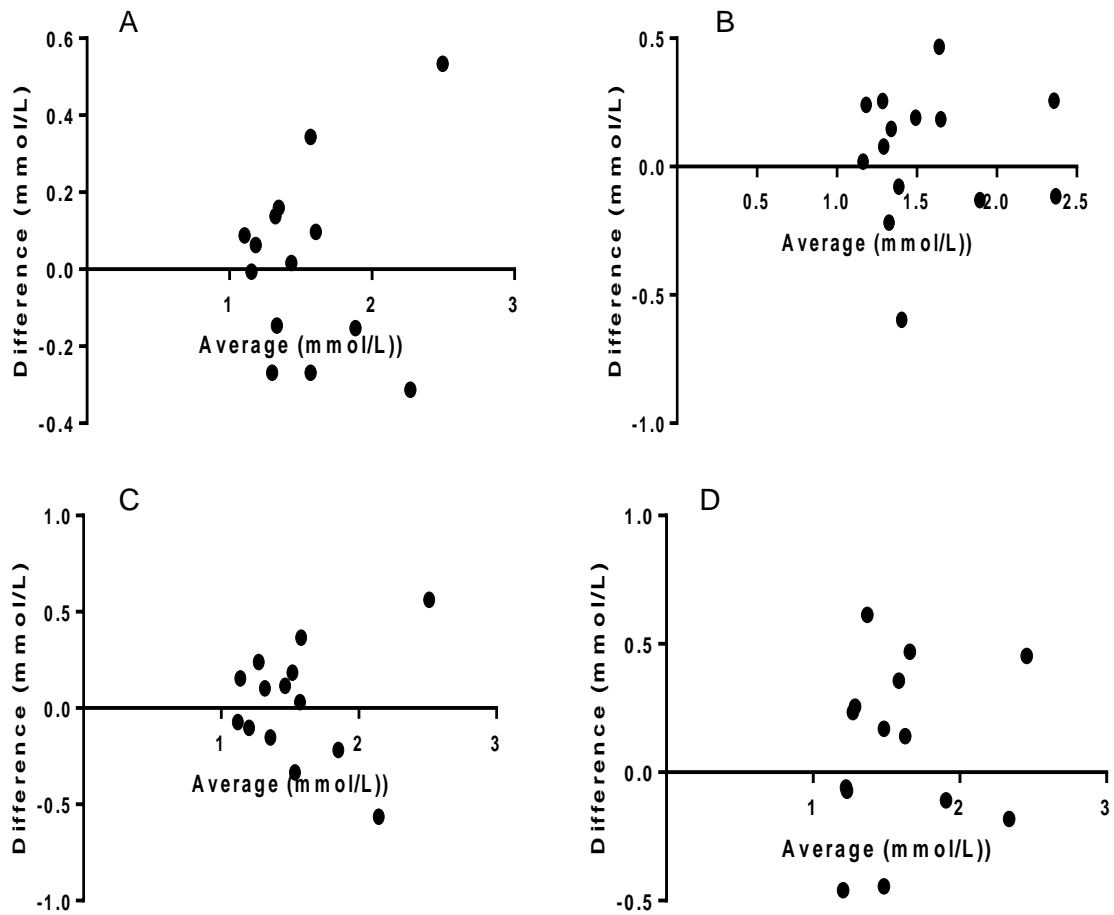


Figure 43: Bland-Altman plot of difference in triglyceride values obtained from fresh plasma and dried plasma spots stored at ambient temperature after 7 days of blood collection, A = Sealed spot, B = Unsealed spot, C = Sealed stored at 4 °C and D = sealed stored at -20 °C

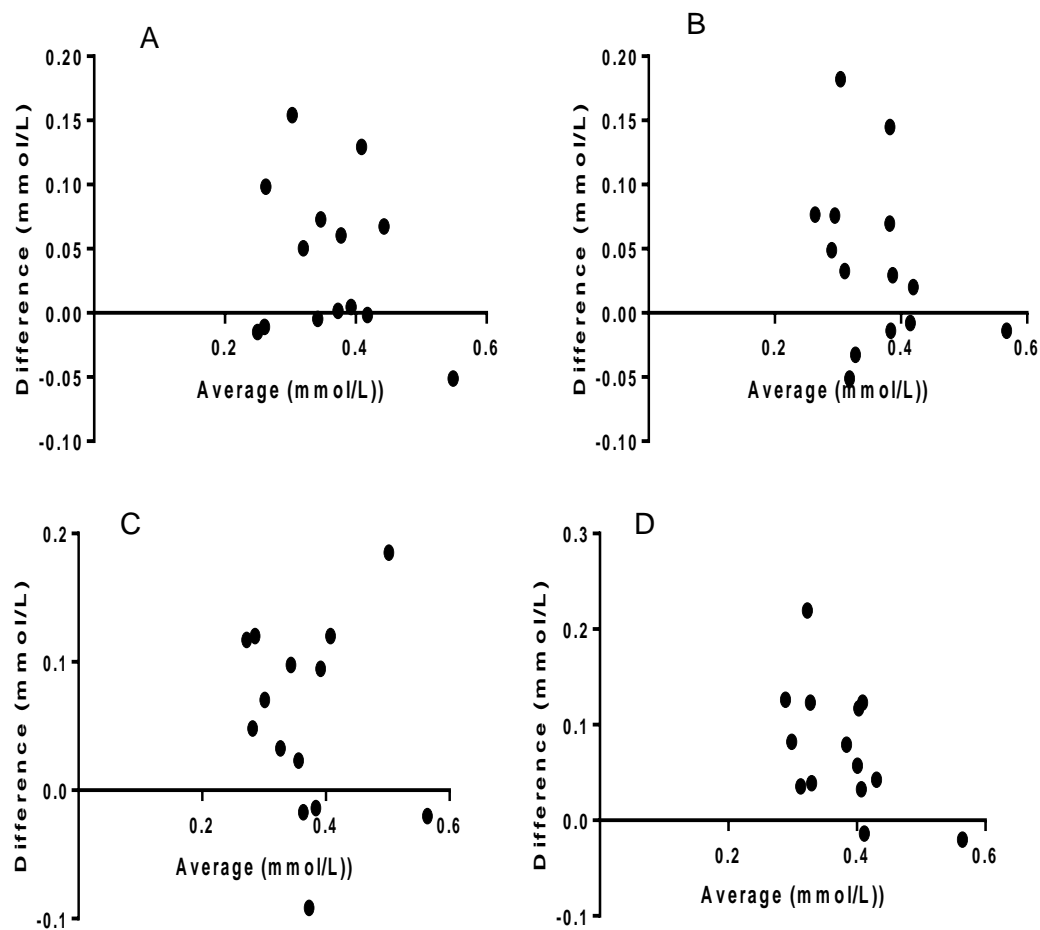


Figure 44: Bland-Altman plot of difference in phospholipid values obtained from fresh plasma and dried plasma spots stored at ambient temperature after 7 days of blood collection, A = Sealed spot, B = Unsealed spot, C = Sealed stored at 4 °C and D = sealed stored at -20 °C

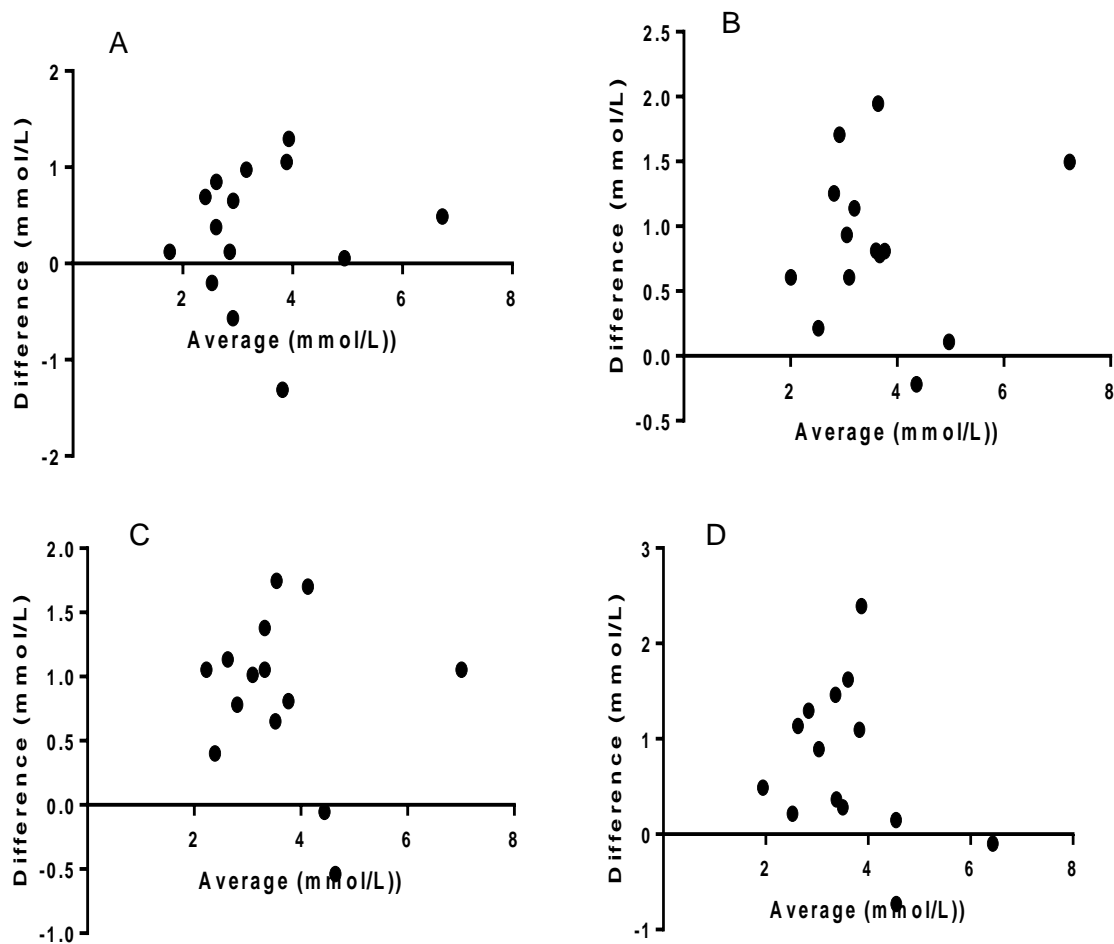


Figure 45: Bland-Altman plot of difference in glucose values obtained from fresh plasma and dried plasma spots stored at ambient temperature after 7 days of blood collection, A = Sealed spot, B = Unsealed spot, C = Sealed stored at 4 °C and D = sealed stored at -20 °C

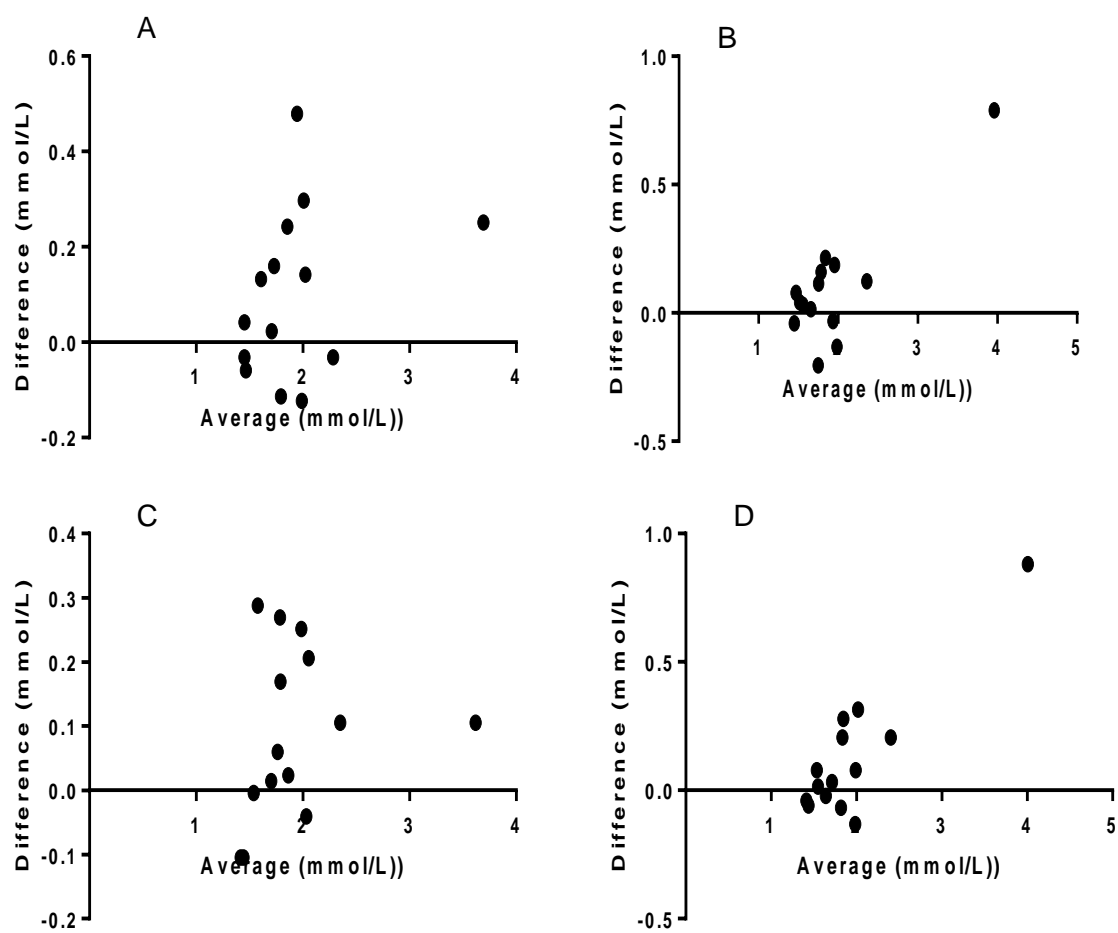


Figure 46: Bland-Altman plot of difference in lactate values obtained from fresh plasma and dried plasma spots stored at ambient temperature after 7 days of blood collection, A = Sealed spot, B = Unsealed spot, C = Sealed stored at 4 °C and D = sealed stored at -20 °C

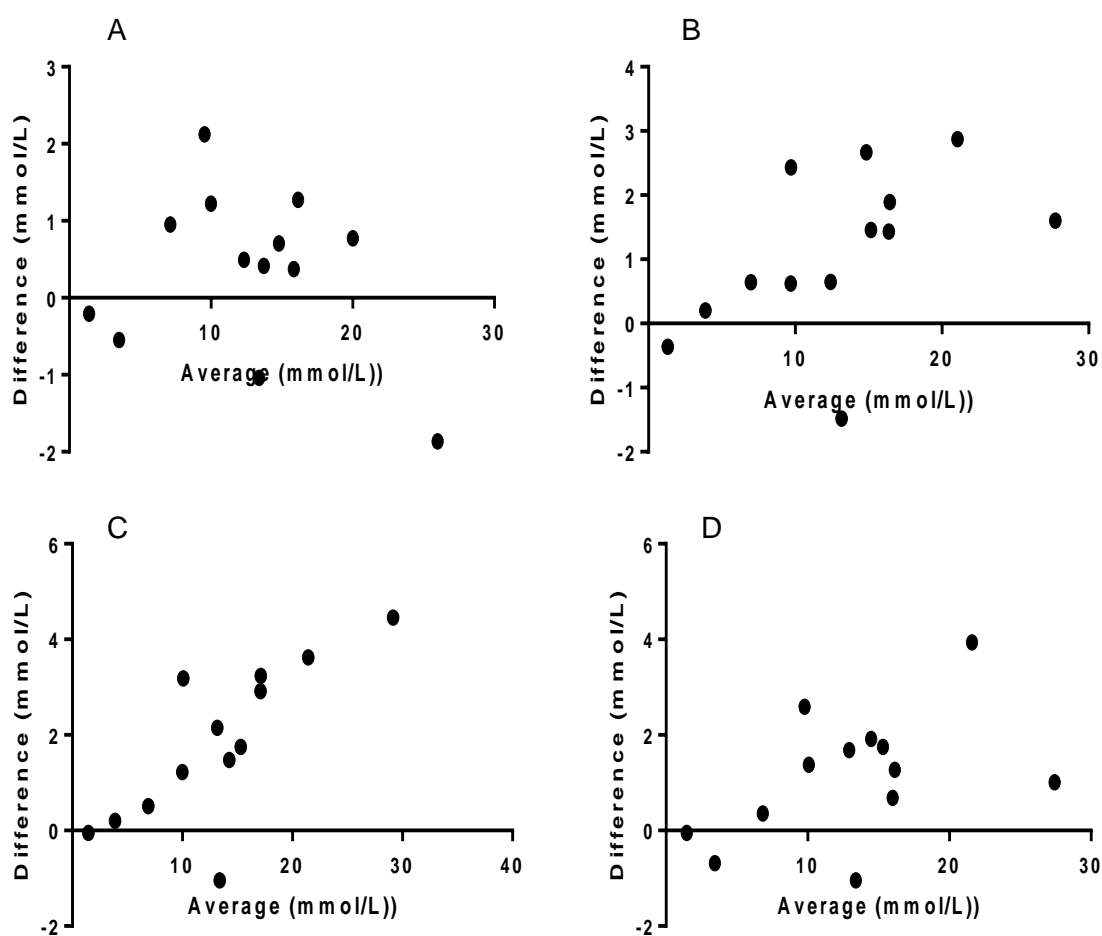


Figure 47: Bland-Altman plot of difference in urine creatinine values obtained from fresh urine and dried urine spots stored at ambient temperature after 7 days of blood collection, A = Sealed spot, B = Unsealed spot, C = Sealed stored at 4 °C and D = sealed stored at -20 °C

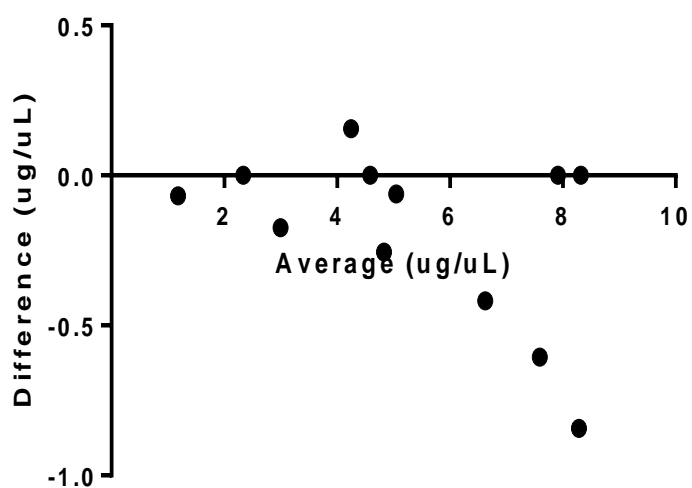


Figure 48: Bland-Altman plot of the difference in salicylate values obtained from fresh serum and dried serum spots after 7 days of blood collection

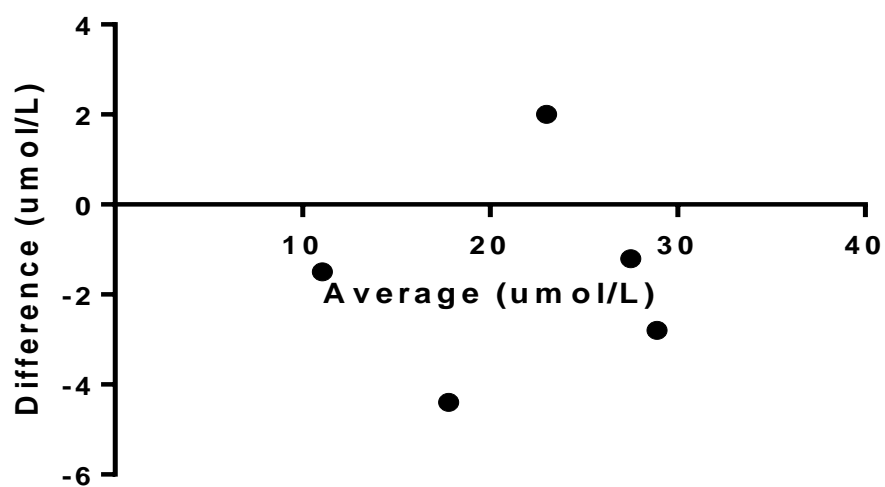


Figure 49: Bland-Altman plot of the difference in cyanide values obtained from fresh blood and dried plasma spots after 7 days of blood collection

Appendix IV

BLOOD						IMD Nr.....
						SURNAME.....
						NAME.....
BUFFY						BIRTH..... F... M...
						Folder.....
						Sample Date.....
PLASMA						Prep Date.....
						Dr.....
PLASMA BUT						Phone.....
						Address.....
					
URINE						PHENOTYPE.....
					
					
URINE					
					
						Bar code:

Figure 50: The layout of the filter paper card as proposed for the collection of biologic fluids

APPENDIX V

Table 2: Comparison of cholesterol, triglyceride, phospholipid, glucose, lactate, protein and urinary creatinine from samples spotted on filter paper after 1 week, with original samples.

Values are median (interquartile range)

	Original samples	Sealed DPS or DUS	Unsealed DPS or DUS	4 °C DPS or DUS	-20 °C DPS or DUS
Cholesterol (mmol/L)	4.44 (3.90 - 4.73)	3.36 (3.02-3.83)	3.30 (2.95 - 3.89)	3.55(3.19-4.15)	3.61(3.32-4.11)
Triglycerides (mmol/L)	1.65(1.24-1.88)	1.46(1.33-1.89)	1.48(1.25-1.82)	1.61(1.32-1.84)	1.67(1.29-1.91)
Phospholipids (mmol/L)	0.40(0.34-0.46)	0.40(0.33-0.45)	0.40(0.35-0.49)	0.38(0.35-0.46)	0.44(0.37-0.47)
Glucose (mmol/L)	2.73(2.36-3.64)	3.89(3.43-4.34)	3.10(2.72-4.46)	3.87(3.41-4.48)	3.93(3.20-4.42)
Lactate (mmol/L)	1.72(1.53-1.98)	2.03(1.71-2.28)	2.01(1.77-2.31)	2.02(1.81-2.26)	1.99(1.71-2.21)
Creatinine (mmol/L)	11.31(6.77-15.55)	12.02(8.87-16.31)	12.08(9.37-15.40)	12.87(9.26-17.68)	12.65(9.33-15.62)
Protein (µg/µL)	89.29(83.37-105.40)	78.65(68.98-84.72)	61.09(55.33-72.23)	73.96(59.10-82.30)	70.16(59.83-82.89)

Appendix VI

Table 3: Comparison of cholesterol, triglyceride, phospholipid, glucose, lactate, protein and urinary creatinine from samples spotted on filter paper after 3 months, with original samples.

Values are median (interquartile range)

	Original samples	Sealed DPS or DUS	Unsealed DPS or DUS	4 °C DPS or DUS	-20 °C DPS or DUS
Cholesterol (mmol/L)	4.23 (3.42 - 5.04)	3.54 (3.07 - 3.85)	3.14 (2.60 - 3.86)	3.54 (3.23 - 4.01)	3.77(3.29-4.18)
Triglycerides (mmol/L)	1.77 (1.43 - 2.34)	1.43 (1.26 - 1.89)	1.59 (1.25 - 1.87)	1.67(1.29-1.91)	1.60(1.25-1.84)
Phospholipids (mmol/L)	0.54(0.45-0.61)	0.40(0.35-0.46)	0.40(0.33-0.46)	0.48(0.45-0.56)	0.52(0.44-0.58)
Glucose (mmol/L)	3.09(2.72-3.90)	3.01(1.91-3.74)	3.35(2.66-4.41)	3.65(2.53-4.74)	3.62(2.93-4.04)
Lactate (mmol/L)	1.93(1.72-2.24)	2.33(2.04-2.51)	2.12(1.90-2.35)	2.22(1.89-2.44)	2.31(1.99-2.66)
Creatinine (mmol/L)	13.24(9.11-16.59)	12.20(8.23-14.09)	12.26(8.81-15.29)	14.28(9.98-18.54)	12.59(5.66-16.59)
Protein (µg/µL)	70.52(64.44-77.09)	48.59(45.75-55.59)	7.90(3.32-11.10)	53.96(48.12-62.76)	55.12(49.94-59.94)

Appendix VII

Table 4: Comparison of cholesterol, triglyceride, phospholipid, glucose, lactate, protein and urinary creatinine from samples spotted on filter paper after 7 months, with original samples.

Values are median (interquartile range)

	Original Plasma	Sealed DPS	Unsealed DPS	4 °C DPS	-20 °C DPS
Cholesterol (mmol/L)	4.41 (3.99 -5.02)	3.94(3.10-4.38)	3.57 (3.10-4.29)	4.25(3.58-4.84)	3.93(3.72-4.24)
Triglycerides (mmol/L)	1.53(1.30-2.10)	1.22(0.82-1.59)	1.08(0.75-1.39)	1.49(1.24-1.90)	1.19(0.68-1.61)
Phospholipids (mmol/L)	0.56(0.50-0.67)	0.27(0.24-0.39)	0.33(0.30-0.39)	0.30(0.24-0.34)	0.28(0.23-0.38)
Glucose (mmol/L)	2.82(2.32-3.52)	2.61(2.09-3.98)	2.79(2.10-3.81)	3.49(2.43-4.25)	3.40(2.60-3.94)
Lactate (mmol/L)	1.78(1.64-1.98)	2.30(2.11-2.56)	2.28(1.79-2.98)	2.45(2.05-2.64)	2.17(1.79-2.64)
Creatinine (mmol/L)	9.96(3.20-11.08)	11.00(8.16-15.19)	10.36(7.31-14.35)	12.15(8.48-15.27)	11.17(6.85-14.87)
Protein (µg/µL)	64.98(52.60-70.76)	42.90(38.08-47.89)	3.82(2.46-6.45)	49.96(36.86-56.87)	47.75(44.38-59.00)

Appendix VIII

Table 5: Summary of coefficients of variation of analyses. One sample was analysed 10 times.

	Liquid samples		Dried samples	
	Intra-assay coefficient (%)	Inter-assay coefficient (%)	Intra-assay coefficient (%)	Inter-assay coefficient (%)
Cholesterol (plasma)	6.0	13.3	7.8	14.3
Triglyceride (plasma)	7.6	7.7	6.0	5.7
Phospholipid (plasma)	11.4	11.7	13.2	13.6
Glucose (plasma)	8.7	6.7	6.3	8.0
Lactate (plasma)	6.8	6.0	4.4	4.8
Creatinine (urine)	11.8	17.0	11.0	10.5
Protein (plasma)	13.4	14.4	13.8	14.9
Salicylate (serum)	7.7	8.6	8.0	7.6
Cyanide (whole blood)	8.8		9.2	

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